SOME EFFECTS OF DIETARY OXALATE ON THE TEETH OF WHITE RATS[1]

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TWO FIGURES

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INTRODUCTION

Previous work in our laboratories (Restarski, Gortner and McCay, '45a; Gortner, Restarski, Bieri and McCay, '45) has shown that the ingestion of solutions of various food acids (phosphoric, citric, lactic, sulfuric) at pH 2.6 caused considerable gross destruction of rat molars in vivo within 1 week. In general the different acid solutions behaved similarly, although there were some differences in the relative severity with which they attacked the teeth.

McClure ('43) first showed that when rats were allowed to consume certain fruit juices or commercial soft drinks, destruction of the enamel occurred. Recently he has reported (McClure and Ruzicka, '46) that citrate solutions, even at neutrality, are capable of producing this result by slowly converting the insoluble calcium salts of the enamel into a soluble calcium citrate complex. In extending our studies to include the effects of oxalic acid and its sodium salts on the

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molars of rats we have obtained effects which are more or less the counterpart of what McClure noticed with citrate. Although oxalic acid is a moderately strong acid, the anion combines with calcium to yield a highly insoluble substance.

METHODS

For this study young white rats, caged singly, were used. They were about 6 to 8 weeks of age and weighed from 75 to 125 gm at the beginning of the experiment. The basal diet employed in most experiments was the open formula dog feed [4] used in previous studies. All diets were fed ad libitum.

When solutions were being tested the daily allowance per rat was 20 ml. All solutions contained 10% of sucrose. The tests usually extended over a 7-day period, although in some cases they were continued for 2 or more weeks when no marked effect on the teeth was observed in the shorter period. The same method as described previously (Restarski, Gortner and McCay, '45b) was used for preparing the jaws and evaluating the effect of the acid solutions on the molars. Sufficient animals were included in each experiment to permit reliable assessment of differences.

EXPERIMENTS AND RESULTS

Oxalic acid solutions

To obtain a solution of pH 2.6 corresponding to acids previously tested, 285 mg of anhydrous oxalic acid were added per liter of solution. Twelve rats were given this solution for variable periods of time: six were sacrificed after 1 week, four more after 2 weeks, and the remaining two animals were killed after 12 weeks. In no instance did the oxalic acid solution etch the teeth. Even when the concentration of oxalic acid was quadrupled (1.14 gm per liter) to give a solution of pH 2.15, no etching was noted on the teeth of any of the eight rats drinking this solution for 3 weeks. Instead a definite opaque deposit was observed on all surfaces of the

[4] GLF dog feed, Canandaigua, New York.

molars which had been exposed above the gum line. This calculus-like encrustation was obtained when oxalic acid or sodium oxalate was included in either the feed or drinking water, the thickness of the deposit varying roughly with the concentration of oxalate and the length of time of ingestion (fig. 1A).

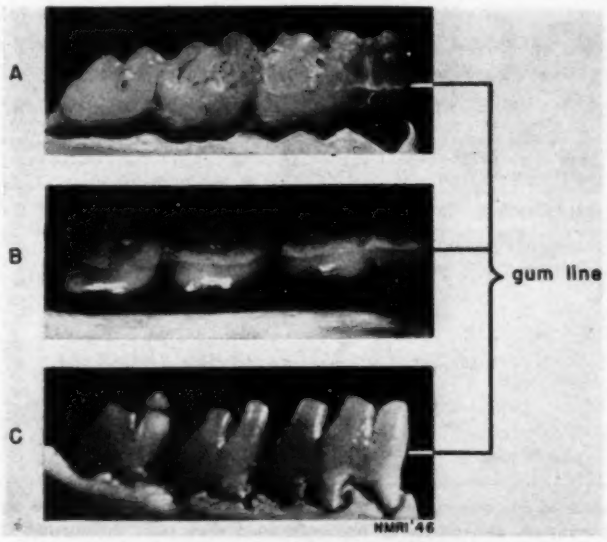

Fig. 1 Heavy calculus-like deposit on rat molars following ingestion of (A) a diet containing 50 mg per cent of sodium oxalate for 12 weeks, and (B) a diet containing 8% of dry spinach for 12 days, compared with normal rat molars (C).

The protective action of oxalate against etching by other acids

It appeared logical that the characteristic film formed by oxalic acid ingestion was protecting the underlying enamel from acid attack. Accordingly, in order to learn whether oxalate might prevent in vivo tooth decalcification by other acids known to cause severe etching by themselves, a series of phosphoric and citric acid solutions was prepared and

oxalic acid was added to some and not to others. In one instance 20 ppm of fluorine (as NaF) was included to see what effect it might have in addition to the oxalate level present.

Eight rats were offered each solution. Half of each group was killed after 1 week, the rest after 2 weeks. The composition of the solutions and the results obtained are shown in table 1.

TABLE 1

The effects of different levels of oxalic acid on the tooth-destructive properties of phosphoric and citric acid solutions when fed to rats over 1- or 2-week periods.

ACID COMPOSITION OF SOLUTIONS			PH OF SOLUTIONS	AVERAGE MOLAR SCORES [1]			
				1 week		2 weeks	
Phosphoric	Citric	Oxalic		Upper jaw	Lower jaw	Upper jaw	Lower jaw
mg %	*mg %*	*mg %*					
55	...	..	2.6	3.2	4.4	3.7	5.2
28	...	14	2.6	2.9	3.5	3.6	4.2
28	...	57	2.3	0.6	0.8	0.3	1.1
55	...	114	2.0	0.0	0.0	0.0	0.0
..	200	..	2.6	2.3	4.6	2.6	5.4
..	200	114	2.0	0.0	0.0	0.0	0.0
28 (plus 20 ppm. F)	...	14	2.6	1.4	1.8	2.5	3.3

[1] A score of zero corresponds to no detectable acid effect; a score of 6 represents almost complete destruction of lingual enamel with some destruction on other surfaces.

It is evident that the oxalic acid afforded protection against decalcification by other acids, being slight at the lowest level and complete at the highest level tried. The effectiveness of fluorine was comparable to what had been obtained in previous studies (Restarski, Gortner and McCay, '45a). To determine more exactly the concentration at which oxalate ion will completely prevent tooth destruction by an acid drink, nine groups of four rats each were offered solutions of a commercially canned orange juice (pH 3.8) to which sodium oxalate was added to give a range of levels corresponding to 0.00–0.15%

oxalic acid. After 1 week the animals were killed and their teeth examined and graded.

In all instances, even in the absence of oxalate, no appreciable etching of the upper molars was seen. This is consistent with observations previously reported (Gortner, Restarski, Bieri and McCay, '45) that citric acid differs from phosphoric acid in that it only mildly attacks the upper molars while severely damaging the lowers.

The data (fig. 2) show that increasing levels of oxalate afforded increasing protection of the mandibular molars up to about the level of 0.1% oxalic acid equivalent. Oxalate

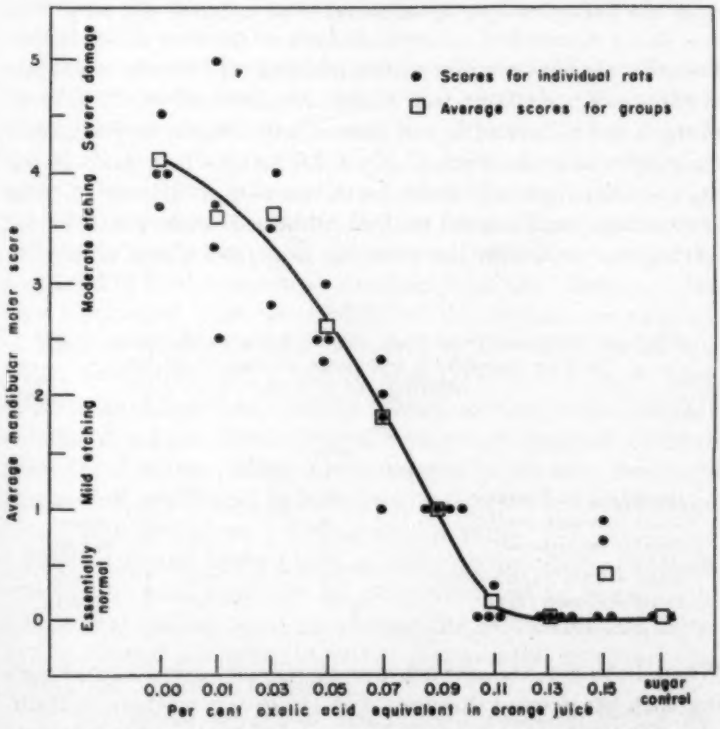

Fig. 2 The effect of different levels of soluble oxalate on the decalcifying action of orange juice on the teeth of rats.

concentrations above this percentage gave essentially complete protection against the orange juice and formed the opaque deposits on the teeth characteristic of oxalate ingestion.

Effects of some natural oxalate containing foods

Certain common foods, notably rhubarb and spinach, are known to contain appreciable levels of oxalate. Accordingly it was of interest to see whether these natural products would act on the teeth similarly to the oxalated feed and beverages studied above.

In the first attempt, equal weights of ground, dry dog feed and finely macerated, canned spinach or thawed, fresh-frozen rhubarb were thoroughly mixed and fed ad libitum to groups of rats over periods up to 4 weeks. No deposits resulted, however, on the molars of any of the animals. When they ingested a phosphoric acid solution of pH 2.6 for the last week of the experimental period, their teeth showed a degree of acid destruction comparable to that obtained with controls receiving the acid solution plus the dog feed alone (table 2).

TABLE 2

Effects of oxalate-containing foods, fed wet or dry, on the etching of rat teeth by a phosphoric acid-sucrose solution of pH 2.6, consumed for 1 week.

DIET	NO. OF RATS	AV. MOLAR SCORE	
Ground dog feed (basal)	12	3.6	Moderately severe damage
Basal + spinach (wet)	8	3.7	
Basal + rhubarb (wet)	8	3.1	
Basal + 0.5% $Na_2C_2O_4$ (dry)	4	0.0	No acid effect
Basal + spinach (dry)	8	0.0	

When, however, the spinach was previously dried before mixing with the ground dog feed diet (to give a mixture containing about 8% of dry spinach), and fed to a group of eight rats over a period of 12 days, the last week of which the

phosphoric acid beverage was also given, the results were as anticipated. A distinct encrustation was found on all molars (fig. 1B) and no etching was apparent (table 2) when the deposit was scraped off and the tooth surfaces examined.

DISCUSSION

The foregoing results serve to show that moderate concentrations of soluble oxalate can effectively inhibit in vivo destruction of rat teeth by acids encountered in foods and beverages. The level of 0.1% oxalic acid (corresponding to a weekly intake per rat of 140 mg of oxalic acid, or the free and combined oxalate in 25 gm of fresh spinach) completely prevented tooth etching by orange juice.

Similar protection against tooth erosion by acids is afforded by natural foods, such as spinach and rhubarb. The petioles of rhubarb are reported (Winton and Winton, '35) to contain the equivalent of about 0.5% oxalic acid, of which approximately three-fifths is in water soluble form. Spinach likewise contains appreciable oxalate in this form. The negative results obtained when these foods were mixed wet with the ground dog feed apparently resulted from the calcium in the feed combining with the oxalate of the spinach or rhubarb so that no soluble oxalate remained in the mixtures as fed. When previously dried before mixing with the stock feed, spinach gave positive oxalate effects on the teeth. Similar results have been obtained by allowing rats to drink rhubarb juice for 1 week. Other foods known to contain moderate amounts of oxalic acid include beet greens, Swiss chard, green beans, taro and cocoa (Winton and Winton, '35).

Buonocore and Bibby ('45), in studying the effect of various ions on the acid solubility of pulverized human enamel, observed that oxalate ion was comparable to fluoride ion in reducing enamel solubility in vitro. Apparently, however, this finding has not been extended.

An interesting aspect of the present research is the observation that soluble oxalates in the food or drink may, over a period of time, build up calculus-like deposits on the teeth

of animals. Presumably this material is crystalline calcium oxalate, resulting from an interaction between oxalate ions and calcium in the saliva. Although human dental calculus is considered to be largely a calcium phosphate complex, it is possible that food oxalate may enter into its composition to a greater or lesser extent.

It should be emphasized that these experiments have been set up to obtain clear-cut effects within a short period of time. The results, therefore, do not justify condemnation of acid foods and beverages for human consumption; indeed some, such as citrus fruits, are valuable dietary constituents, even though they may well have some long range influence on the dentition of man.

Observations of this nature, however, may throw some light on human dental problems, such as tooth erosion and, perhaps, caries formation. It is a general observation of dentists that caries are seldom found under calculus deposits. Furthermore the results already obtained suggest that, at practicable levels, oxalate is more effective than fluoride in preventing in vivo dissolution of enamel by acids. In accordance with these observations, studies are now underway to determine whether levels of dietary oxalate sufficiently low to minimize tooth deposits and interference with calcium utilization will materially protect against caries development in the cotton rat.

The observations of Jones, Larsen and Pritchard ('30) on dental disease in Hawaii are of interest in this regard. They reported that the old Hawaiians in the rural districts, who clung to their old diets consisting primarily of taro (poi) and fish, had excellent teeth, but that the substitution of refined foodstuffs (polished rice, patent flour, etc.) in the diet of the urban groups was followed by obvious dental deterioration. Since taro has been reported to contain about 0.49% free and combined oxalic acid (Winton and Winton, '35) and the old Hawaiians consumed 5 pounds or more of poi daily, it is obvious that the diet was unusually high in oxalate content. How much of this was in a soluble form is problematical.

It is recognized that oxalic acid in the diet may have some influence on the utilization of calcium. The extensive literature existing on the toxicity and metabolism of oxalate in various animals and man is, however, largely outside the scope of this report. Suffice it to say that if the oxalate intake is appreciably great and the dietary calcium level is low, growth and bone formation may be definitely depressed.

In some of our experiments, when rats were maintained on a low calcium diet[5] (either with or without small amounts of added sodium oxalate) for periods of 1 to 4 months, their growth was poor and at autopsy their bones were found to be soft and weak. On the other hand, the addition of as much as 0.5–1.0% of sodium oxalate to the dog-feed diet did not noticeably affect the growth or bone strength of rats consuming this diet for 4 weeks, suggesting that the stock feed had at least a moderately high calcium content. This is consistent with the observation of Mackenzie and McCollum ('37) that the growth and bone ash of rats receiving a diet optimal in calcium, phosphorus and vitamin D were unaffected when 0.9% of potassium oxalate was fed over 10 weeks. Even 2.5% of the oxalate had no effect on growth or gross calcium excretion, and had little effect on the bone ash.

The recent work of Mitchell and Smith ('45), who studied the calcium balance of a group of seven women students receiving diets low or borderline in calcium (225 to 700 mg per day), showed that no deleterious effects could be detected when cocoa was fed up to levels of 35 gm daily. Since cocoa contains 0.5–0.6% oxalic acid, the highest level would correspond to 175 mg of oxalic acid daily. Others (Bonner et al., '38) have reported that even when 700 mg of oxalic acid were added daily to the diet of children receiving 800 to 1300 mg of food calcium, no adverse effect on the calcium utilization was apparent. Thus, it appears probable that certain foods containing moderate levels of soluble oxalate may be safely

[5] Degerminated white corn meal, 76%; corn oil, 10%; dried brewer's yeast, 5%; corn germ, 5%; cod-liver-oil, 3%; salt (NaCl), 1%.

included in the human dietary at average levels of calcium intake.

SUMMARY

1. Small amounts of soluble oxalate in the food or drink produced a hard deposit, which grossly resembled human dental calculus, on the teeth of rats. The extent of this encrustation, which is presumably calcium oxalate, varied with the concentration of oxalate consumed and with the length of the experiment.

2. Because of this phenomenon, oxalic acid solutions having a pH as low as 2.1 did not, in vivo, etch the enamel of the teeth of rats, in contrast to the action of other common food acids (citric, lactic, phosphoric, sulfuric) at even higher pH levels.

3. When oxalic acid or its sodium salt was present in the rat's food or drink the decalcification of teeth, which accompanies ingestion of phosphoric (0.055%) and citric (0.20%) acid solutions, diminished or disappeared. Essentially complete protection against the etching action of orange juice was afforded by a concentration of 0.1% oxalate in the juice.

4. Natural oxalate-containing foods, such as spinach and rhubarb, when incorporated into the diet in a manner which did not remove the soluble oxalate prior to ingestion, produced the characteristic protective films on the molars of rats within 1 week.

5. Inasmuch as these experiments with rats were devised to give clearcut results within a short time, caution must be exercised in interpreting these data with respect to human practices. It is hoped, however, that further studies using foods with moderate levels of "soluble" oxalate may offer some insight into the dietary control of dental calculus and caries initiation.

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DEPLETION OF HEPATIC RESERVES OF VITAMIN A AND CAROTENE IN CATTLE[1]

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TWO FIGURES

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Various investigators have reported on the time required to produce symptoms of vitamin-A deficiency in cattle on rations low in carotene. Dickson, Jones and Schmidt ('35) and Guilbert and Hart ('35) have shown that night blindness is one of the first readily detectable clinical manifestations of vitamin-A deficiency to appear in cattle. Halverson and Sherwood ('30) produced night blindness in steers in 88 days on a ration of cottonseed hulls and meal while dairy heifers of about 1 year of age, on the same ration, required from 150 to 250 days to produce night blindness. Mead and Regan ('31) produced signs of vitamin-A deficiency in calves in 1 to 3 months after changing the ration from whole milk and grain to one low in vitamin A. Riggs ('40) has shown that range cattle from 3 to 16 months of age, on a ration practically devoid of carotene, will develop night blindness in from 46 to 266 days. The variability in time required to produce night blindness was shown to be dependent upon the age of the animal and the nature of the carotene-deficient ration as well as the ration fed previously.

Little information is available concerning the rate of depletion of hepatic reserves of vitamin A and carotene in cattle

[1] Journal Paper no. 200, Colorado Agricultural Experiment Station.

during the period of depletion of the body reserves of these two constituents. Guilbert and Hart ('35) have reported on the body reserves of vitamin A and carotene in cattle which had access to rations containing an abundant amount of carotene, and also on cattle which had the body reserves of the two constituents depleted to such an extent as to result in night blindness. Guilbert et al. ('37) have reported minimum vitamin-A and carotene requirements for cattle using night blindness as a test for minimum requirement.

The present paper deals with the rate of depletion of initially large vitamin-A and carotene reserves in the livers of steers while in the feed lot during an experimental period of 166 days. Two dietary groups are involved. One group was maintained on a ration high in carotene and the other on a ration relatively low in carotene. The data presented herein were obtained in one phase of a study designed to determine the cause of liver abscess formation in cattle.

EXPERIMENTAL

The animals used in the present experiment consisted of 140 Hereford steers of approximately 18 months of age at the beginning of the experiment. The steers were selected from a single herd and placed in the feed lot on November 9, 1944. The steers had been on native grass pasture in Jackson County, Colorado, since the previous spring and for several weeks prior to being placed in the feed lot had received a protein supplement of about 1 lb. of cottonseed cake daily.

Twenty-two animals were slaughtered at the beginning of the experiment and the remaining animals divided into two dietary groups. One dietary group of 98 animals was placed on a fattening ration as shown in table 2. The carbohydrate content of the ration was gradually increased during the feeding period of 166 days. All ration constituents, except salt and mineral supplement, were hand fed. The second dietary group of twenty animals was placed on a maintenance ration as shown in table 2, all self fed. The corn silage and cottonseed meal were gradually decreased as ration con-

stituents until at the end of 40 days the ration consisted entirely of alfalfa hay, salt and mineral supplement. Records were kept on consumption of ration constituents for both dietary groups. Random lots of animals were slaughtered at approximately 40-day intervals as shown in table 1.

Samples of liver tissue were obtained at the time each animal was slaughtered. The samples were sealed in glass jars containing dry ice and kept frozen until analyzed. Vitamin A was determined colorimetrically (Carr and Price, '26; Davies, '33) by means of an Aminco type-F photoelectric colorimeter. Crystalline vitamin-A alcohol was used as the reference standard. The vitamin A and carotene were extracted

TABLE 1

Slaughtering data.

DAYS IN FEED LOT	NUMBER OF ANIMALS SLAUGHTERED	
	Fattening ration	Maintenance ration
0	22	
41	19	
76	20	
119	19	10
166	40	10

from the saponified liver with petroleum ether and dried over anhydrous sodium sulphate. The carotene was determined colorimetrically in the dried petroleum ether extract. Beta-carotene, dissolved in petroleum ether, was used to obtain the standard curve on the colorimeter.

The average values found for vitamin A and carotene for each group of animals slaughtered are shown in figure 1. One liver sample was lost from the group on the maintenance ration slaughtered after 166 days in the feed lot. Otherwise, the number of livers analyzed is as given in table 1.

The ration constituents were analyzed for carotene content at frequent intervals and the carotene intake per animal per day calculated. The values so obtained are summarized in

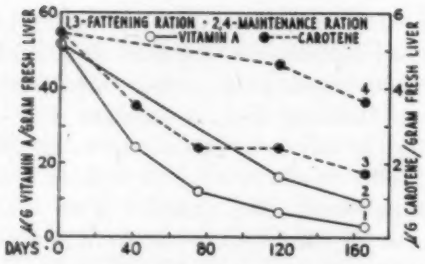

Fig. 1 Graphical representation of depletion of liver vitamin A and carotene reserves of steers with days in the feed lot. Expressed as micrograms of constituent per gm of fresh liver.

TABLE 2

Average daily ingestion of ration ingredients in pounds and the calculated carotene intake per animal.

FEEDING INTERVAL IN DAYS	ALFALFA HAY	CORN SILAGE	GROUND CORN	BRAN	COTTON SEED MEAL	CAROTENE, IN $\mu G \times 10^3$
			Fattening ration			
0– 41	8.14	6.14	9.11	0.00	1.25	469
41– 76	4.89	5.01	14.98	0.72	1.00	317
76–119	3.76	3.72	16.63	0.96	0.95	237
119–166	3.17	2.69	18.27	0.99	0.98	205
			Maintenance ration			
0– 41	15.96	6.60	0.00	0.00	0.50	883
41– 76	20.13	0.00	0.00	0.00	0.00	1030
76–119	17.23	0.00	0.00	0.00	0.00	878
119–166	20.07	0.00	0.00	0.00	0.00	1060

table 2, each value representing an average over a feeding period of approximately 40 days.

DISCUSSION

The data given herein include values obtained from both normal livers and those showing a pathological condition. The only pathological conditions found were telangiectasis, abscess and "sawdust." Barron ('42) has reported variations in the vitamin-A content of the liver as the result of various pathological conditions. Unpublished data by the present

authors show no significant variation in the vitamin-A content of the liver as a result of the pathological conditions observed in the animals under investigation.

A definite trend is shown in the vitamin-A and carotene values for the animals on the fattening ration throughout the entire feeding period of 166 days (fig. 1, curves 1 and 3). There is some indication of a similar trend in the vitamin-A values for the animals on the maintenance ration (fig. 1, curve 2). Depletion of initially large reserves of both constituents are indicated in the curves cited, the rate of depletion diminishing as the feeding period progressed. The carotene intake for the animals on the maintenance ration was relatively high compared to the carotene intake for the animals on the fattening ration (table 2). This would account for the high level of carotene reserve maintained by the animals on the maintenance ration (fig. 1, curve 4). The above is in agreement with the work of Baumann, Riising and Steenbock ('34) who showed that the rate of depletion of vitamin A in the liver of the rat decreases with time as the liver reserve of vitamin A becomes smaller.

The average amount of carotene ingested daily by the animals on the maintenance ration was 963 mg (table 2). The largest total liver reserve of carotene found in any of the experimental animals was 50 mg. This would indicate a daily requirement of carotene by cattle considerably in excess of the total hepatic storage in order to maintain the liver reserve of carotene.

No clinical evidence of avitaminosis A was observed in any of the animals during the experimental period. Consistent gains in weight were obtained for the animals in both dietary groups. Guilbert et al. ('37) obtained excellent gains in weight in cattle with a very low storage of vitamin A. Jones et al. ('38) found young Hereford steers to become very fat on a ration practicaly devoid of carotene even though the animals were totally night blind. The average vitamin-A value of the livers of the animals on the fattening ration, after 166 days in the feed lot, was 1.9 μg of vitamin A per gm of fresh

liver (fig. 1). This would indicate that hepatic reserves of vitamin A may fall to an extremely low level in cattle without producing acute symptoms of avitaminosis A.

A comparison of the percentages of the hepatic reserves of vitamin A and carotene retained in each of the two dietary groups, after 166 days in the feed lot, shows that the carotene reserves of the liver are more readily maintained than are the vitamin A reserves (table 3). At the beginning of the experimental period there was present in the liver 9.68 μg of

TABLE 3

Initial and final hepatic reserves of vitamin A and carotene (see fig. 1) expressed as micrograms of constituent per gram of liver.

	VITAMIN A	CAROTENE
INITIAL RESERVE	51.4	5.31
	Fattening ration	
Final reserve	1.9	1.80
Per cent of initial reserve	3.7	33.9
	Maintenance ration	
Final reserve	9.4	3.77
Per cent of initial reserve	18.3	71.0

vitamin A for each microgram of carotene (fig. 2). After 166 days in the feed lot this value had dropped to 1.06 μg for the animals on the fattening ration and to 2.50 μg for the animals on the maintenance ration. Braun ('45) reported vitamin-A and carotenoid values of the blood and liver of cattle with and without vitamin-A supplement with a view to establishing a relationship between vitamin-A levels of the blood and liver. When vitamin-A and carotenoid values for individual livers were compared he found the ratio $\frac{\text{vitamin A}}{\text{carotenoid}}$ to decrease with increasing carotenoid levels of the liver. Reference to figure 2 will show increasing values for the ratio $\frac{\text{vitamin A}}{\text{carotene}}$ of the liver with increasing carotene levels of the liver.

The values for the ratio $\frac{\text{carotene content of liver}}{\text{daily carotene intake}}$ show no significant variation (table 4). This would seem to indicate that the carotene reserves of the liver vary in direct proportion to the carotene intake.

The values for the ratio $\frac{\text{vitamin A content of liver}}{\text{daily carotene intake}}$ show successively decreasing values (table 4). The decreasing values for the ratio indicate an increasing rate of loss of hepatic reserves of vitamin A with decreasing carotene intake. Corresponding ratios for the animals on the maintenance ration are not given since the animals were on a fairly constant carotene intake.

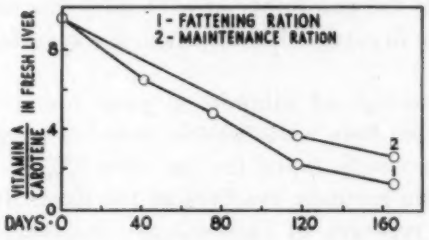

Fig. 2 Graphical representation of the decrease in the $\frac{\text{vitamin A}}{\text{carotene}}$ ratio of the liver with days in the feed lot. Expressed as micrograms of constituent per gm of fresh liver.

TABLE 4

The ratios $\frac{\text{carotene content of liver}^1}{\text{daily carotene intake}}$ *and* $\frac{\text{vitamin A content of liver}^1}{\text{daily carotene intake}}$ *for the animals on the fattening ration. Daily carotene intake given in grams (table 2) and liver constitutents in micrograms per gram of fresh liver.*

FEEDING INTERVAL IN DAYS	CAROTENE CONTENT OF LIVER / DAILY CAROTENE INTAKE	VITAMIN A CONTENT OF LIVER / DAILY CAROTENE INTAKE
0– 41	7.65	50.5
41– 76	7.66	37.5
76–119	10.00	22.4
119–166	8.78	9.3

[1] Values for carotene and vitamin A, as found at the end of the feeding interval, were used in calculating the above ratios.

SUMMARY

The rate of depletion of initially large hepatic reserves of vitamin A and carotene is given for 140 steers over an experimental period of 166 days. The animals were divided into two dietary groups. One group was on a fattening ration low

in carotene and the other on a maintenance ration relatively high in carotene. The rate of depletion of the hepatic reserves of vitamin-A and carotene decreased as the liver reserves of the two constituents decreased.

No clinical evidence of avitaminosis A was observed in any of the animals throughout the experimental period. This would indicate the possibility of extremely low levels of vitamin A reserve in cattle without producing symptoms of avitaminosis A.

Hepatic reserves of vitamin A were found to be more readily depleted than were hepatic reserves of carotene. Increasing values were found for the ratio $\frac{\text{vitamin A}}{\text{carotene}}$ of the liver with increasing carotene reserves of the liver. It was found that hepatic reserves of carotene are maintained in cattle in direct proportion to the carotene intake. An increasing rate of loss of hepatic reserves of vitamin A occurred with decreasing carotene intake.

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RIBOFLAVIN EXCRETIONS OF YOUNG WOMEN ON DIETS CONTAINING VARYING LEVELS OF THE B VITAMINS[1]

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ONE FIGURE

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Although a number of studies have been made on the excretion of riboflavin by persons maintained on a controlled intake of the vitamin, only a few have had women as subjects. Sebrell, Butler, Wooley and Isbell ('41) maintained a group of ten women on a diet containing 0.21 mg riboflavin per 1000 Cal. for a period of 4 to 8 months. The daily excretion averaged 74 μg riboflavin per day. Six of the women developed cheilosis. On the basis of added amounts of the vitamin required to prevent or cure cheilosis and of the urinary excretion on different levels of intake, these workers suggested that the riboflavin requirement was about 3 mg/day, or about 1.2 to 1.5 mg/1000 Cal. The results of a brief experiment on four college girls by Strong, Feeney, Moore and Parsons ('41) appeared to confirm this recommendation. On a dietary intake of 1 to 2 mg riboflavin the daily urinary excretion was 50 to 150 μg, in comparison to a 500 to 800 μg daily excretion observed in a group of students and faculty members eating

[1] The expenses of this study were defrayed by funds granted by the National Dairy Council on behalf of the American Dairy Association and by the Williams-Waterman Fund of the Research Corporation.

[2] The data reported in this paper were taken from a thesis presented by Margaret V. Davis to the Faculty of the Division of Biological Sciences of the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

their normal diet. More recent work indicates that these early recommendations were high, however. The daily urinary excretion of riboflavin and the response to a test dose of the vitamin by groups of women maintained on different levels of riboflavin intake were studied by Williams, Mason, Cusick and Wilder ('43). Urinary excretion fell to about 100 μg/day and test dose returns were markedly lowered on an intake of 0.35 mg/1000 Cal. On an intake of 0.5 mg/1000 Cal., daily excretion fell to low levels but test dose returns were not affected as greatly as on the smaller amount. On an intake of 0.8 mg/1000 Cal. there was no noticeable decrease in the excretions. These workers suggested that the requirement of riboflavin by women was not less than 0.5 mg, nor more than 0.8 mg/1000 Cal., or a total of 1.0 to 1.6 mg per day.

In the present work the riboflavin excretion of a group of healthy young women, living on a diet in which the amount of riboflavin was progressively increased, was followed over a period of 8 months. The young women were university students, leading a life characteristic of such a group. Efforts were made to insure that the experimental procedures should result in as little change as possible in their usual habits. It was hoped that such a study would yield further information on the utilization of and requirement for riboflavin by normal young women, insofar as such requirements can be judged on the basis of excretion of the vitamin on known levels of intake. That the excretion level of a vitamin reflects the tissue concentration of that vitamin has been demonstrated in the case of thiamine by Hulse and co-workers ('44).

METHODS OF STUDY

Altogether twelve young women between the ages of 19 and 32 years served as subjects during the course of the study. Of the nine original subjects, three were followed throughout the entire 8-month period, the others for periods of 3½ to 5 months. Three months before the end of the study it was decided that at the level of riboflavin intake planned for the remainder of the time, new subjects might be included after

a short period of adjustment to the diet. Three young women were selected to serve as subjects at this time, together with three who had been on the diet since the beginning.

Each subject received a complete physical and neurological examination some time during the first 2 weeks of the study.[3] This examination was repeated after a period of 7 weeks on the experimental diet. At the end of 3½ months a special examination was made for any signs of riboflavin deficiency.

The results of the preliminary examinations showed the young women to be in a good general state of health. Two persons (subjects 7 and 10) were found to have hemoglobin values somewhat below normal, and three deviated considerably from the average weight for their height. Subject 2 was 17% overweight, while subjects 1 and 3 were approximately 20% underweight. There were no other negative findings.

The experiment was divided into five periods. During the first period, which lasted for 106 days, the average intake of riboflavin was 0.29 mg/1000 Cal. After the first 59 days on this level certain changes were made in the diet which increased the intake of thiamine, but did not change that of riboflavin. The times before and after this change have been designated as periods I-a and I-b. In period II, 45 days in length, the riboflavin intake was increased to an average of 0.49 mg/1000 Cal., and in period III, which lasted 41 days, to an average of 0.66 mg/1000 Cal. In period V, 14 days long, the diet was supplemented with 6 mg riboflavin, 4 mg thiamine, 0.5 mg pantothenic acid, and 10 mg niacin per day. This period of "supercharging" was followed by a return, for 20 days, to approximately the same level of intake as in period III.

Except during period IV, when the supplements of synthetic vitamins were given, and during periods III and V when a daily supplement of approximately 100 μg of thiamine was given, all variations in the vitamin content of the diet were made through changes in the kinds and amounts of foods.

[3] Grateful acknowledgment is made to Dr. Oscar Kreisler, who kindly made these examinations.

This was done because it was considered that the results would be more applicable to practical situations than if the synthetic vitamin were used.

The menus for the experimental diet were worked out on the basis of 3-day periods. The same foods were served each 3 days, although their method of preparation was varied.

TABLE 1

Amounts of basic foods served every 3 days during each experimental period.

FOOD	PERIOD				FOOD	PERIOD			
	I-a	I-b	II	III and V		I-a	I-b	II	III and V[1]
	gm	gm	gm	gm		gm	gm	gm	gm
Beef	150	150	200	200	Rhubarb	...	...	100	...
Chicken	75	75	100	100	Berries	...	...	...	100
Carrots	70	70	100	100	Grapefruit juice	120	120	...	...
Beets	70	70	...	...	Tomato juice	120	...	120	120
Green beans	60	60	100	100	Apple juice	120	...	...	...
Peas	...	...	100	100	Orange juice	...	200	360	360
Potatoes	100	100	300	300	Rice	200	200	...	...
Head lettuce	100	100	190	210	Farina	340	...	...	...
Celery	30	30	20	...	Oatmeal	...	300	400	400
Pineapple	72	72	200	200	Cream	<132	<110	<110	<110
Apple	200	200	100	...	Milk	...	...	600	1050[2]
Peaches	...	...	100	100	Jam	<150	<150	<150	<150
Pears	...	...	...	100					

[1] The same general dietary pattern was followed during period IV, when supplements were given, as during periods III and V. Some variation was allowed in period IV, however.

[2] In an attempt to keep vitamin/calorie ratios similar for all subjects, subject 2 received 600 gm of milk every 3 days, and subject 12, 900 gm every 3 days during periods III and V.

Foods which contained considerable amounts of riboflavin, such as meat, milk, fruits and vegetables, were served in equal quantities to each person. The amounts of these foods served in each 3-day period will be found in table 1.

In order to increase the caloric intake, cake and cookies and usually some form of pastry were served in each 3-day period in approximately equal amounts to each person. Bread and

butter were allowed ad libitum as required to meet individual caloric needs. All of the baked products were prepared in the laboratory using unenriched flour, low vitamin yeast, water, and minimal amounts of egg.

A complete record of food consumption was kept for each individual. No food was eaten except that served at meals, although outside consumption of certain beverages was permitted. A careful record of these was kept by each subject, and they were included when the caloric value of the diet was calculated.

The basal diet was planned to be low in both riboflavin and thiamine. The niacin content of the diet in the early periods was only slightly below the present recommended allowance of 12 mg per day for moderately active women. It was assumed that the diet was adequate in other B-vitamins for which requirements are not known. Supplements of di-calcium phosphate, ferric pyrophosphate, vitamin A and ascorbic acid were given. Complete details of the nutritive value of the diet and of its supplementation will be found in table 2. The amount of iron given in period I-b was increased over that in period I-a in connection with a study on serum iron which was made on the subjects during period I.

Preliminary observations on the average daily excretion of riboflavin, the fasting 1-hour excretion, and the return of a test dose were made during the week prior to the start of the experimental diet. The subjects continued on a self-chosen diet during this week, except that they were asked not to eat liver or pork during the period.

During the course of the experiment 72-hour urine collections were made each week, except during the first week of a new experimental period. The urine samples were collected in brown glass bottles, and preserved with acetic acid, toluene, and a few drops of chloroform. One-fifth of the entire 3-day sample was held under refrigeration until analyzed.

During the last week in each experimental period two 1-hour specimens of urine voided before breakfast were collected. On one morning during this week a low-vitamin breakfast

TABLE 2

Length of periods, number of subjects, and composition of the diet throughout the study.

	PERIOD					
	I-a	I-b	II	III	IV	V
Length (days)	59	47	45	41	14	20
Number of subjects	9	9	5	6	6	6
Average intake [1]						
Riboflavin — mg/1000 Cal.	0.29	0.28	0.49	0.66	4.10 [4]	0.63
Thiamine — mg/1000 Cal.	0.14	0.20	0.36	0.51 [3]	2.80 [4]	0.54 [3]
Niacin — mg	11.0	9.7	10.6	14.3	F [5] 14.3 S 10.0	14.3
Pantothenic acid — mg	2.3	2.4	3.5	4.3	F 4.3 S 0.5	4.7
Protein — gm	49	51	61	62	62	62
Fat — gm	73	78	82	68	68	68
Carbohydrate — gm	311	307	289	247	247	247
Vitamin A — I.U. [2]	F 4200 S	F 4200 S	F 6300 S 5000	F 6500 S 5000	F 6500 S 5000	F 6500 S 5000
Ca — gm	F 0.17 S 0.35	F 0.22 S 0.24	F 0.47 S 0.24	F 0.67 S 0.24	F 0.67 S 0.24	F 0.67 S 0.24
Fe — mg	F 6.9 S 4.0	F 7.6 S 8.0	F 10.8 S ..	F 11.3 S ..	F 11.3 S ..	F 11.3 S ..
Vitamin C — mg	F42.4 S50.0	F70.8 S50.0	F113.4 S 25.0	F115.3 S 25.0	F115.3 S 25.0	F115.3 S 25.0

[1] Figures for riboflavin, thiamine, nicotinic acid and pantothenic acid determined by analysis; those for other nutrients by calculation.

[2] International Units.

[3] Figure includes supplement of approximately 100 μg thiamine per day.

[4] Figure includes supplements — 6 mg riboflavin, 4 mg thiamine.

[5] F — amount supplied by food; S — amount supplied by supplement.

was given, and the urine excreted during the following 4 hours was collected. This constituted a "4-hour control sample." On the next to last morning of each period a test dose of riboflavin was given orally with the same low-vitamin breakfast and a 4-hour sample collected.[4] The size of the test dose was 0.02 mg/kg body weight, which gave a total of approximately 1 mg. In calculating the percentage of the test dose which was returned, the riboflavin value of the 4-hour control sample was subtracted from that of the 4-hour sample taken following the test dose.

During the times when urine collections were being made, samples of all foods were saved for analysis. Composites representing one-fifth of the total amount of food taken by each person, with the exception of cereal foods, sugar, and butter, were made at the end of the 3-day periods. The samples were homogenized, brought to approximately pH 1 by the addition of 8 ml concentrated hydrochloric acid per liter of suspension, and autoclaved at 15 pounds pressure for 15 minutes. Cereal foods were analyzed separately and the amount of riboflavin taken by each person in such foods was determined by calculation. This procedure was followed in order that the cereals would not exert any untoward effects on the riboflavin analyses.

Fecal collections, marked by carmine, representing the same period as the last 3-day urine collection in each period except period IV were made by some of the subjects. After the wet weight had been recorded, the feces were placed in a large evaporating dish and covered with 50% alcohol. Succeeding samples were added to the dish, which was kept refrigerated until the collection was complete. The samples were dried to constant weight at 45°C. After drying they were ground and stored in the refrigerator until analyzed.

The riboflavin content of the food composites, urine, and feces was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42). A modified

[4] Riboflavin was generously supplied by Merck and Company.

basal medium, the same as that used by Oldham, Johnston, Kleiger and Hedderich ('44) for cereal analyses, was used for all analyses in this study.

RESULTS AND DISCUSSION

A summary of the results of the study is found in table 3. Included in this table are the results of (1) the preliminary measurements of urinary excretion of riboflavin, (2) the average daily intake and urinary excretion of the vitamin by each subject during each period, and (3) the average daily fecal excretion, fasting 1-hour excretion and return of a test dose at the end of each period.

Results of preliminary observations. As would be expected among a group of people of different dietary habits, there was considerable variation in the daily urinary excretion of riboflavin during the preliminary period. There appeared to be a relationship between the daily excretion and the amounts of riboflavin-containing foods, particularly of milk, taken by the young women. Subjects 7 and 11, who had the lowest daily excretions, drank little milk. All of the others were accustomed to drinking milk in varying amounts.

Only four of the subjects returned as much as 20% of the test dose, the amount which was suggested by Oldham and co-workers ('44) as indicative, in the case of children, of a satisfactory nutritional status with regard to riboflavin. One of these four returned approximately 35%, which is the amount suggested by Feder, Lewis and Alden ('44) as a normal return of a test dose. It should be noted, however, that in the data presented by Feder et al., the total 4-hour excretion following the administration of the test dose is used in calculating the percentage returned. In the method of calculation used in this paper, where the normal 4-hour excretion is subtracted from the amount excreted in the 4 hours following the test dose, a smaller percentage return would be expected. Although the return of a test dose has been used as a criterion in nearly every study of riboflavin requirements, the size of the test dose, the method of its administration, and

of calculating the return have varied. Because of these variations it is difficult to assess the original nutritional status of the subjects on the basis of test dose returns.

Twenty-four-hour excretions

The 24-hour excretions of riboflavin adjusted to changes in intake quite rapidly. After about 10 days on a new dietary level, the daily excretions reached approximately the level at which they remained throughout the period. One exception was observed during period I-a. This was subject 2, whose excretion of riboflavin during the preliminary period was much higher than that of anyone else. Her riboflavin excretion dropped gradually during the first 6 weeks on the experimental diet and by the seventh week had reached a level at which it continued until the end of period I-b. In the case of subjects 7 and 11, who had shown low daily excretions of riboflavin during the preliminary period, the 24-hour excretion level did not change significantly when the experimental diet was started.

In spite of the relatively large increase in riboflavin intake in period II, from 0.29 to 0.49 mg/1000 Cal., there was only a small increase in the daily excretion of the vitamin. However, when the riboflavin intake was increased in period III to 0.66 mg/1000 Cal., there was a definite rise in excretion in all subjects. As would be expected, an immediate, striking rise in daily excretion occurred in period IV when the diet was supplemented with 6 mg riboflavin. Urine collections were made during the first 3 days and last 3 days of this period. An average of 55% of the total intake of riboflavin was excreted during the first 3 days, and of 66% during the last 3 days. Apparently there was a tendency for the body to "hold on" to some of the extra vitamin at first. Subject 2 excreted a greater proportion of the supplement than did the others; this indicates, perhaps, that her stores were more nearly full than were those of the other subjects.

Following the vitamin supplementation, and at the end of 2 weeks on an average daily intake of 0.63 mg/1000 Cal., the

TABLE 3

Average daily intake and excretion of riboflavin by each subject throughout the experiment together with average daily fecal excretion, fasting 1-hour excretion and test dose return by each subject at the end of each experimental period.[1]

SUBJECT	1	2	3	4	5	6	7	8	9	10	11	12	AVERAGE
HEIGHT, CM	165	169	161	170	160	152	164	175	165	158	174	170	
WEIGHT, KG	46.3	71.7	45.8	56.2	56.2	50.8	58.5	62.6	59.0	59.4	63.5	69.4	
Preliminary period													
Excretion													
Urinary													
μg/24 hr.	480	931	305	617	283	508	145	628	421	190	109	581	433
Fasting													
μg/hour	8	34	17	34	20	13	8	25	12	15	8	22	18
Test dose													
% return	13.3	34.8	20.2	13.0	15.9	23.1	11.8	...	8.2	13.1	10.6	19.6	16.7
Period I-a													
Intake													
Total, μg	631	555	539	561	608	...	658	671	610	...	633	...	616
Mg/1000 Cal.	0.29	0.33	0.27	0.30	0.29		0.29	0.27	0.29	...	0.27	...	0.29
Excretion													
Urinary													
μg/24 hr.	114	183	88	124	80	...	129	127	97	...	98	...	119
Fecal													
μg/24 hr.	....	....	478	598	872	..	794	...	..	....	...	...	686
Fasting													
μg/hour	7	11	7	6	7	...	7	8	8	...	6	...	8
Test dose													
% return	0.3	11.6	3.3	3.2	2.2	...	2.6	1.0	1.0	..	0.1	..	2.8
Period I-b													
Intake													
Total	585	529	534	572	523	..	649	629	566	...	625	...	585
Mg/1000 Cal.	0.27	0.30	0.23	0.27	0.29		0.27	0.27	0.27	...	0.26		0.28
Excretion													
Urinary													
μg/24 hr.	107	125	84	95	82	...	142	115	92	...	97	..	107
Fecal													
μg/24 hr.	407	399	573	512	587	..	..	..	..	..	..	..	496
Fasting													
μg/hour	6	8	8	4	4	...	4	5	6	..	6	...	6
Test dose													
% return	7.0	11.6	5.0	2.9	1.5	...	4.3	0.8	3.3	..	4.0	...	4.5
Period II													
Intake													
Total	1070	967	1013	1056	981	...	..	...	...	..	...	...	1017
Mg/1000 Cal.	0.48	0.57	0.47	0.44	0.51	...	...	...	...	...		...	0.49
Excretion													
Urinary													
μg/24 hr.	179	185	141	142	101	...	...	...	...	...	...	...	150
Fecal													
μg/24 hr.	259	778	647	614		...	...	...	...	..	...	...	575
Fasting													
μg/hour	12	9	13	12	8	...	...	...	...	...	...		11
Test dose													
% return	18.4	10.5	9.7	11.5	8.8	...	...	...	...	...	...	...	11.8

TABLE 3 — *(continued)*

SUBJECT HEIGHT, CM WEIGHT, KG	1 165 46.3	2 169 71.7	3 161 45.8	4 170 56.2	5 160 56.2	6 152 50.8	7 164 58.5	8 175 62.6	9 165 59.0	10 158 59.4	11 174 63.5	12 170 60.4	AVERAGE
Period III													
Intake													
Total	1360	1134	1244	...	...	1347	...	...	...	1253	...	1063	1234
Mg/1000 Cal.	0.65	0.64	0.65	...	...	0.63	...	...	...	0.68	...	0.72	0.66
Excretion													
Urinary μg/24 hr.	345	214	234	...	...	287	...	...	...	229	...	269	263
Fecal μg/24 hr.	415	656	570	..	...	657	...	...	...	...	...	574	574
Fasting μg/hour	8	11	15	...	...	14	...	...	...	8	...	9	11
Test dose % return	26.5[2]	17.9	12.9	...	...	13.9	...	...	...	11.1	...	15.5	14.3
Period IV													
Intake, total													
Week 1	7252	7071	7210	...	...	7213	..	...	..	7169	..	7066	7164
Week 2	7257	7045	7232	...	...	7254				7210		7065	7177
Excretion, urinary/24 hr.													
Week 1	4275	4835	3968	...	...	4254	..	..	...	3361	..	2917	3935
Week 2	4847	5805	4958		...	4538				4547	...	3823	4753
Period V													
Intake													
Total	1291	1086	1254	..	...	1275	...	...	..	1267	...	1065	1206
Mg/1000 Cal.	0.59	0.54	0.68			0.59			...	0.59	.	0.76	0.63
Excretion													
Urinary μg/24 hr.	403	504	299	..	...	243	...	...	...	212	...	286	325
Fecal μg/24 hr.	361	856	611	...	...	628		..	...	....	...	540	599
Fasting μg/hour	16	15	14	...	...	10	..	..	..	16	..	11	13
Test dose % return	18.1	17.0	10.2	..	...	24.6		...	..	14.6	...	9.1	15.6

[1] Daily intakes and urinary excretions during periods I-a, I-b, II, and III represent the average of 3-day collections made weekly throughout these periods, with the exception of those for subject 3 in periods I-a and I-b which represent a single 3-day collection at the end of these periods.

[2] This figure is not included in the average. Due to an error the 4-hour collection was not made following the test dose, and a second test dose was given the following day. This is the return of the second test dose.

daily urinary excretion was almost exactly the same as it had been during period III for five of the six subjects. Here again subject 2 was the exception, her daily excretion being almost double that observed in period III. It should be pointed out that the results shown for period V represent only one 3-day collection. Throughout the course of the experiment there were a few cases in which the average daily excretion for a particular week did not fall exactly in line with the results secured during the rest of the period. It is possible that something of this nature may have occurred in the case of this particular collection. In view of the other results secured on subject 2, however, it seems probable that she did have more riboflavin "available" for excretion than did the other subjects.

Fasting 1-hour excretion

During periods I-a and I-b, when the average intake was 0.29 mg/1000 Cal., the fasting 1-hour excretion dropped sharply, and at the end of period I-b it averaged 6 μg. When the riboflavin intake was increased to an average of 0.49 mg/1000 Cal., the fasting excretion rose to an average of 11 μg per hour. There was no further increase in the fasting excretion when the intake was increased to 0.66 mg/1000 Cal. in period II, nor was the excretion in period V (13 μg per hour) following the supplementation in period IV, appreciably higher than in period III.

The fasting 1-hour excretions were also calculated on a unit volume basis as suggested by Feder, Lewis and Alden ('44). It was found that the values for excretion per hour were more constant than the values for excretion per unit volume.

Test dose returns

A sharp drop in test dose returns was found to have occurred at the end of 59 days on an average intake of 0.29 mg/1000 Cal. At that time four of the nine subjects excreted 1% or less of the test dose, while four others returned between 2 and 4%. One person, subject 2 again, showed a much higher

return than anyone else in the group — 11.6%. At the end of period I-b the average return of a test dose was slightly higher, despite the fact that there had been no increase in the amount of riboflavin in the diet. If subject 1, who showed an unusually large increase in test dose return (from 0.3 to 7.0%) is not included, the average returns are 3.1 and 4.2% for periods I-a and I-b, respectively, which still indicate a slight increase. Although the level of riboflavin in the diet was not changed at the end of period I-a, the thiamine was increased from an average of 0.14 to 0.20 mg/1000 Cal. It is possible that this increase in thiamine may have had some effect on the utilization of riboflavin.

At the end of period II there was a sharp increase in the amount of the test dose returned, the average at this time being 11.8%. There was a further increase, to an average of 14.3%, at the end of period III. Apparently no additional benefit was derived from the 2-week period of vitamin supplementation; the average amount returned at the end of period V was 15.6%, practically the same as the average return at the end of period III. Two of the six subjects showed an increased return; the other four returned approximately the same amount or slightly less than in period III. According to the theory of Melnick ('42) this would indicate that the tissue stores of riboflavin had been satisfactorily filled on the 0.66 mg/1000 Cal. level of intake, since the 2-week period of high intake did not affect the test dose return.

Fecal excretion

Although there was considerable variation in the total amount of riboflavin excreted in the feces by different individuals, the level for each person remained relatively constant. Apparently the fecal excretion was not influenced either by changes in the dietary level of riboflavin, or by the fact that the starch content of the diet was higher during the early periods of the study, when larger amounts of cereal foods were necessary to maintain the caloric intake.

That intestinal synthesis of riboflavin did occur is shown by the fact that in some cases the fecal excretion alone was higher than the dietary intake of the vitamin. In a number of other cases the total urinary and fecal excretion was higher than the intake. Whether any absorption or utilization of this synthesized vitamin took place is difficult to determine.

General health

No definite signs or symptoms of riboflavin deficiency were observed at the time of the original examination, or in the examination at the end of period I-a. No cheilosis was observed among any of the subjects at any time, nor did any seborrheic dermatitis occur. The eyes were not examined with a slit lamp, but a magnifying glass failed to reveal any abnormal vascularization of the cornea. During the first examination the tongue of one of the young women (subject 3) was observed to show slight enlargement and reddening of the fungiform papillae, and some indentation of the margin. This condition did not change throughout the study. When the special examination of the mouth, tongue, and eyes was made at the end of period I-b, two other subjects were observed to have a similar condition of the papillae, and one showed slight indentations of the margin of the tongue. Two subjects were found to have slight photophobia when the eyes were exposed to a strong light. One of these was subject 11, whose daily excretion and return of a test dose were low. The other was subject 2, whose excretions were high. All of the changes were slight, however, and the clinician who made the examination stated that they were "not convincing and of doubtful significance."

Constipation was observed by certain of the subjects during periods I-a and I-b, and was severe in several cases. This occurred in spite of the fact that fruit juice, raw or cooked fruit, and a cooked and a raw vegetable were included in the meals each day. The condition was relieved immediately when the diet was changed in period II, at which time both the riboflavin and thiamine contents of the diet were increased.

At the end of period I-b four young women withdrew from the study because of unfavorable subjective symptoms. All had severe constipation and all complained of fatigue, lack of concentration, desire to sleep, and general malaise. It seems likely, however, that these symptoms, if caused by the diet, were due to a low intake of thiamine rather than of riboflavin.

Interpretation of results

A graphic comparison of the average intake and excretion of riboflavin by all of the subjects throughout the course of the study is found in figure 1. From the pronounced drop in

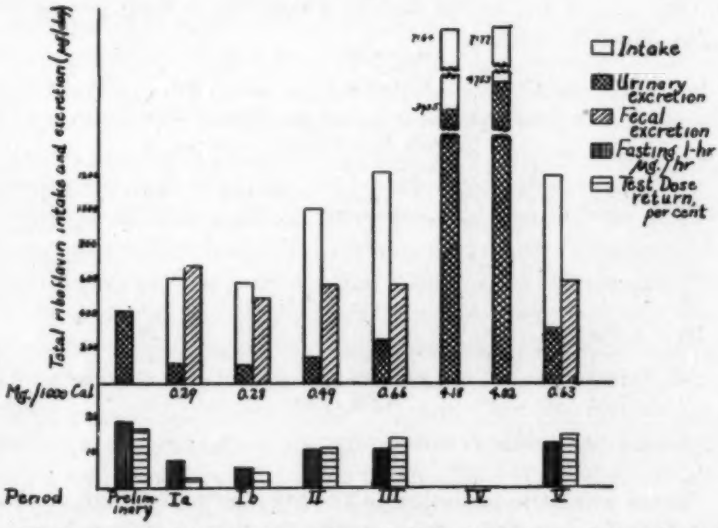

Fig. 1 Average intake and excretion of riboflavin during each experimental period.

daily excretion, fasting excretion, and test dose returns which occurred during period I, when the riboflavin content of the diet was restricted to 0.29 mg/1000 Cal., it seems evident that the riboflavin in the body tissues reached a low level during this period.

Although the 24-hour excretion did not increase greatly when the riboflavin intake was raised to an average of 0.49 mg/1000 Cal., there was a striking increase in both the fasting excretion and the amount of a test dose returned. The data on the test dose returns indicate that the concentration of tissue riboflavin had been increased during this period.

The rise in 24-hour excretion in period III (on an average intake of 0.66 mg/1000 Cal.) was greater in proportion than might have been expected from the dietary increase in riboflavin. The average increase in riboflavin intake in each period, together with the resulting increase in excretion are shown in table 4. The percentage excreted, both of the total intake and of the added dietary riboflavin in each period is also shown in table 4.

TABLE 4

Percentage of the total intake and of the increase in intake which was excreted at each dietary level.

PERIOD	TOTAL INTAKE	INCREASE IN INTAKE	TOTAL EXCRETION	INCREASE IN EXCRETION	TOTAL INTAKE EXCRETED	INCREASE IN INTAKE EXCRETED
	μg	μg	μg	μg	%	%
I	600		113		19	
II	1017	417	150	37	15	9
III	1234	217	263	113	21	52
IV	7171	5937	4344	4081	61	69
V	1206		325		27	
V[1]	1230		289		23	

[1] Average if subject 2 is not included.

There was little variation in the average percentage of the total intake excreted, except during the period of supplementation. The figures on the percentage of total intake excreted probably are not so significant, however, as those on the percentage of the added riboflavin which was excreted in each period. When the riboflavin intake was increased from an average of 0.29 to 0.49 mg/1000 Cal. in period II (a total increase of about 400 μg per day) nearly all of the added riboflavin was "retained" by the body, only 9% of the added

amount being excreted. When 200 μg per day were added in period III to make an intake of 0.66 mg/1000 Cal., more than 50% of the added amount was excreted. It seems logical to assume that when a relatively small increase in riboflavin intake results in an excretion of more than 50% of that increase, the added amount was not necessary.

The fact that both the daily urinary excretion and the test dose returns were increased when the average intake was 0.66 mg/1000 Cal. in period III, together with the fact that there was no apparent benefit from a 2-week period of vitamin supplementation, would indicate that this level of riboflavin intake probably is more than enough to take care of bodily needs for the vitamin. The observation that despite the low daily excretion of riboflavin, the fasting 1-hour excretion and the return of a test dose rose on an intake of 0.49 mg/1000 Cal. suggests that this level of intake supplies at least the minimal "requirements." This seems even more evident when it is considered that this level of intake followed a long period of low intake during which there was evidence that the tissue concentration of riboflavin had been decreased. This amount of riboflavin is the same as that found by Williams and his co-workers ('43) to supply the minimal requirements of riboflavin for women. This evidence, together with the data presented herewith which indicate that an intake of 0.66 mg/1000 Cal. supplied more of the vitamin than was necessary, strongly suggests that the riboflavin "needs" of healthy, active young women can be met by an intake of about 0.50 mg/1000 Cal., or a total intake of approximately 1 mg.

SUMMARY

The riboflavin excretion of young women on a diet in which the amount of the vitamin was progressively increased was followed during an 8-month period.

From an initial intake during periods I-a and I-b of 0.29 and 0.28 mg/1000 Cal., the riboflavin level was increased by

the addition of milk and by other dietary changes to 0.49 and 0.66 mg/1000 Cal. during periods II and III. In period IV, which lasted 2 weeks, the total daily intake was 7.1 mg. During period V, 3 weeks long, the intake averaged 0.63 mg/1000 Cal.

Physical examinations at the beginning of the experiment and following periods I-a and I-b revealed no signs of riboflavin deficiency.

Daily urinary excretions "levelled off" within 10 days after each change in diet. Average daily excretions during the first four periods were 119, 107, 150, and 263 μg, respectively. Excretions rose sharply during supplementation, but after 2 weeks on a lower intake the average excretion was 325 μg.

Levels of fecal excretion of riboflavin differed considerably among individuals, but the amount excreted by each person remained relatively constant despite dietary variations.

Test dose returns at the end of periods I-a and I-b averaged 2.8 and 4.5%. With increased intake in periods II and III, the average returns were raised to 11.8% and 14.3%, respectively. Test dose returns were not increased by supplementation, the average at the end of period V being 15.6%.

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THIAMINE EXCRETIONS AND BLOOD LEVELS OF YOUNG WOMEN ON DIETS CONTAINING VARYING LEVELS OF THE B VITAMINS, WITH SOME OBSERVATIONS ON NIACIN AND PANTOTHENIC ACID[1]

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ONE FIGURE

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Studies of the thiamine requirement of women based on the presence or absence of deficiency symptoms have been made by Elsom ('42), and Williams ('40, '42, '43a, '43b) and their associates. Elsom and her co-workers ('42) reported that six subjects who received a daily intake of less than 600 μg all developed clinical signs of deficiency and had daily urinary excretions of 42 μg or less. Three other subjects maintained on 650 to 775 μg per day showed no signs of deficiency for approximately a month and had urinary excretions of 46 to 60 μg. They concluded that the minimal thiamine intake compatible with health was about 650 μg and the thiamine-calorie ratio, about 0.35. Williams and his associates ('40, '42, '43b), who have made an extensive series of studies, have repeatedly reported definite signs of clinical deficiency, low urinary excretions and low test dose returns when women were maintained on daily intakes of less than 0.40 mg per 1000 cal. In an additional long-time experiment on five subjects who were receiving intakes of 0.45 mg per 1000 cal. these authors ('43a)

[1] The expenses of this study were defrayed in part by funds granted by the National Dairy Council on behalf of the American Dairy Association and by the Williams-Waterman Fund of the Research Corporation.

found some increase in blood pyruvate following glucose administration in all but one subject and depletion of tissue stores as evidenced by daily urinary excretions of less than 100 μg and test dose returns of less than 20% in some subjects. They have concluded that 0.45 mg of thiamine per 1000 cal. cannot be regarded as representing more than the minimal requirement.

Melnick ('42) analyzed the daily urinary excretions and test dose returns of a large number of individuals, both men and women, on different thiamine intakes. He concluded that a daily intake of 1 mg or 0.35 mg per 1000 Cal. resulted in excretions indicative of adequate tissue stores. However, since he believed that a margin of safety was desirable, he recommended an intake of 0.50 mg per 1000 cal.

Approximately the same value was obtained by Foltz, Barborka and Ivy ('44) as the result of a study of young men where both work output and urinary excretions were used as criteria. In the series of experiments conducted by Keys and his associates ('43, '45), however, no evidences of physical impairment and no decrease in work output was observed when young men were maintained for several months on intakes of 0.23 mg per 1000 Cal., although the urinary excretions and test dose returns were low. On a still lower intake of 0.185 mg per 1000 Cal. these authors found no signs of deficiency except an elevation in the resting level of blood pyruvate and a slight but similar tendency after work. The daily urinary excretions on this level of intake averaged only 7 μg during the last 3 weeks of the study. It is worthy of note, however, that the total thiamine intakes of Keys' men, because of their higher caloric intakes, were much higher than those of the smaller, less active women subjects of Williams and Elsom.

The present study, which was carried out in conjunction with one on riboflavin reported by Davis, Oldham and Roberts ('46), was undertaken in order to secure additional information on the amount of thiamine necessary to maintain reasonable tissue concentrations of this vitamin in young women

as evidenced by thiamine excretions and blood levels. That changes in the tissue concentration of thiamine are reflected by such measurements has been demonstrated by Hulse and co-workers ('44). These authors found definite decreases in the yeast stimulating activity of skeletal muscle and blood and in the urinary excretion of thiamine of six young men on a low thiamine diet as compared to that on their self-selected diets.

The intakes and excretions of niacin and pantothenic acid were also determined throughout the study, although at less frequent intervals than those of thiamine.

METHODS OF STUDY

This study was carried out in conjunction with the one on riboflavin. The general plan and the subjects have been described elsewhere by Davis et al. ('46). Therefore, only a brief review will be given here.

After a preliminary period of 7 days in which the urinary excretions of thiamine, the blood levels and the returns of test doses were determined on a freely chosen diet, the experiment ran continuously for 226 days. This was divided into four main periods which varied in length from 41 to 59 days. They were followed by a 14-day period of supplementation after which there was a 20-day period on intakes which were approximately the same as those previous to the supplementation. Diets were given which were adequate except for their riboflavin and thiamine contents. The intakes of these vitamins were low in the first period and were gradually increased as the study progressed. The thiamine levels amounted to 0.14, 0.20, 0.36 and 0.51 mg per 1000 Cal. and the riboflavin levels to 0.29, 0.28, 0.49 and 0.66 mg per 1000 Cal. in the first four periods. Daily supplements of 6 mg of riboflavin, 4 mg of thiamine, 10 mg of niacin and 0.5 mg of pantothenic acid were added in the next period and in the final period the intakes of thiamine and riboflavin were 0.54 and 0.63 mg per 1000 Cal., respectively.

Seventy-two-hour food composites and urine collections were made weekly. At the end of each period one or more

1-hour fasting urine collections and two 4-hour urine collections, one before and one after the administration of a test dose of thiamine, were made. Samples of blood were also taken at the end of each period.

The kinds and amounts of foods served, their preparation, the supplements of nutrients other than the B vitamins, the adequacy of the diet, the method used in making composites of individual intakes for analyses and the collection and treatment of urinary and fecal excretions have been reported by Davis et al. ('46). The administration of the oral test dose of 20 μg of thiamine [2] per kg of body weight was similar to that of the riboflavin test dose but was always given the day after the riboflavin test dose in order that the excretion of riboflavin would not be affected (Klopp, Abels and Rhoads, '43). The method of calculation of the test dose return wherein a correction was made for the basal excretion by subtracting a 4-hour control sample, was the same as that used in the case of riboflavin.

The food composites, fecal collections, and all urine samples except the fasting 1-hour specimens in certain periods were analyzed for their thiamine content by the method of Hennessy and Cerecedo ('39) as modified by Najjar and Ketron ('44). The fasting 1-hour specimens in all but the last two periods were analyzed by the microfermentation method of Atkin, Schultz and Frey ('39), as described by Knott, Kleiger and Torres-Bracamonte ('43), because of their extremely low thiamine content. The blood samples were also analyzed by the latter method.

The 72-hour food composites representing the last week of each period, except the one in which the supplements were given, and the corresponding urinary and fecal collections were assayed for nicotinic acid by the microbiological method of Krehl, Strong and Elvehjem ('43) and for pantothenic acid [3] by the method of Neal and Strong ('43) with certain

[2] Thiamine was generously supplied by Merck and Company.

[3] During the periods in which pantothenic acid was determined, separate aliquots of urine collections and food composites were prepared by autoclaving without the addition of acid and preserving with toluene.

modifications.[4] In the supplemented period analyses were made for both the first and the last 3 days.

RESULTS AND DISCUSSION

Urinary excretions of thiamine

The thiamine content of the diets at each level of intake were found to be quite constant and the daily excretions adjusted within a week to changes in intake. Therefore, only average results are presented for each period. The data for individual subjects are summarized in table 1. The average results for the group are shown in figure 1. The data on fecal thiamine excretions and those on niacin and pantothenic acid are presented in tables 2 and 3, respectively, and will be discussed separately.

In the preliminary period, on a freely chosen diet, none of the subjects was considered to be in more than a fair state of nutrition with respect to thiamine as judged by the usual standards of interpretation of excretion data. The average daily excretion was 97 μg and the average test dose return, 6.3%. Four subjects, moreover (7, 8, 9 and 11) were considered to be in distinctly poor nutritional states. The daily excretions of three of this group (7, 8 and 11) were 38, 56 and 35 μg, and the test dose returns of three (8, 9 and 11) were 3.7, 2.3 and 2.7%, respectively. A careful physical examination by an experienced clinician,[5] however, revealed no deficiency symptoms.

In period 1-a on an average daily intake of 295 μg or 0.14 mg per 1000 Cal., the daily excretions of thiamine ranged from 16 to 31 μg and the blood levels showed a definite decrease in every case. The test dose returns averaged 1.5% with only one subject returning over 2.0%. During this period four subjects (7, 8, 9 and 11) complained of severe con-

[4] Two hundred μg each of thiamine, pyridoxine, niacin and para-amino benzoic acid, 4 μg of biotin and 10 mg each of adenine, guanine and uracil were used in each liter of basal medium. The vitab supplement was omitted.

[5] We are indebted to Dr. Oscar Kreisler for the physical examinations.

TABLE 1

Urinary thiamine excretions and blood levels on different intakes.[1]

SUBJECT HEIGHT, CM WEIGHT, KG	1 165 46.3	2 169 71.7	3 161 45.8	4 170 56.2	5 160 56.2	6 152 50.8	7 164 58.5	8 175 62.6	9 165 59.0	10 158 59.4	11 174 63.5	12 170 69.4	AVERAGE
Preliminary period													
Excretion:													
Urinary μg/24 hr.	194	150	126	75	95	171	38	56	107	127	35	150	97 [2]
Fasting μg/hour	5	8	8	6	6	...	2	5	6	5	6	4	6 [2]
Test dose % return	9.8	12.0	5.5	8.2	7.1	11.9	5.8	3.7	2.3	9.0	2.7	6.0	6.3 [2]
Blood level μg/100 ml	5.7	5.0	6.4	4.4	5.0	...	4.0	5.4	5.3	6.3	5.7	6.7	5.2 [2]
Period I-a (59 days)													
Intake:													
μg/24 hr.	298	251	288	262	290	...	325	337	294	...	308	...	295
Mg/1000 Cal.	0.14	0.15	0.15	0.14	0.14	...	0.14	0.13	0.14	...	0.13	...	0.14
μg/kg	6.4	3.5	6.3	4.7	5.2	...	5.6	5.4	5.0	...	4.9	...	5.2
Excretion:													
Urinary μg/24 hr.	27	26	22	18	16	...	16	31	21	...	20	...	22
Test dose % return	3.7	0.4	1.5	0.3	1.4	...	1.8	1.0	1.7	...	1.5	...	1.5
Blood level μg/100 ml	3.3	3.3	4.5	3.7	2.6	...	3.6	5.0	3.6	...	4.5	...	3.8
Period I-b (47 days)													
Intake:													
μg/24 hr.	427	395	402	424	391	...	459	462	415	...	441	...	424
Mg/1000 Cal.	0.20	0.23	0.17	0.20	0.21	...	0.19	0.20	0.20	...	0.19	...	0.20
μg/kg	9.2	5.5	8.8	7.5	7.0	...	7.8	7.4	7.0	...	6.9	...	7.5
Excretion:													
Urinary μg/24 hr.	49	24	19	36	25	...	39	38	32	...	38	...	33
Fasting μg/hour	6	4	6	4	6	...	4	3	4	...	8	...	5
Test dose % return	0.7	1.3	0.8	0.1	0.4	...	0.3	0.4	1.3	...	0.6	...	0.7
Blood level μg/100 ml	5.7	4.9	5.0	3.7	5.5	...	4.3	6 6	5.7	...	6.7	...	5.3
Period II (45 days)													
Intake:													
μg/24 hr.	768	702	764	761	714	...	...	...	...	...	...	...	742
Mg/1000 Cal.	0.35	0.41	0.35	0.32	0.37	...	...	...	...	...	...	...	0.36
μg/kg	16.6	9.8	16.7	13.5	12.7	...	...	...	...	...	...	...	13.9
Excretion:													
Urinary μg/24 hr.	71	53	35	35	42	...	...	...	...	...	...	...	47
Fasting μg/hour	8	5	7	6	6	...	...	...	...	...	...	...	6
Test dose % return	7.6	4.7	2.3	1.0	3.2	...	...	...	...	...	...	...	4.0
Blood level μg/100 ml	7.2	6 6	7.1	6.3	6.6	...	...	...	...	...	...	...	6.8

TABLE 1 (*continued*)

Urinary thiamine excretions and blood levels on different intakes.[1]

SUBJECT HEIGHT, CM WEIGHT, KG	1 165 46.3	2 169 71.7	3 161 45.8	4 170 56.2	5 160 56.2	6 152 50.8	7 164 58.5	8 175 62.6	9 165 59.0	10 158 59.4	11 174 63.5	12 170 69.4	AVERAGE
Period III (41 days)													
Intake:													
µg/24 hr.	962	883	952	...	...	979	...	...	...	949	...	896	937
Mg/1000 Cal.	0.47	0.55	0.49	...	...	0.49	...	...	...	0.52	...	0.54	0.51
µg/kg	20.8	12.3	20.8	...	...	19.3	...	...	...	16.0	...	12.9	17.0
Excretion:													
Urinary µg/24 hr.	139	80	77	...	...	144	...	...	...	135	...	68	107
Fasting µg/hour	9	12	5	...	...	7	...	...	...	3	...	2	6
Test dose % return	10.4	5.2	6.1	...	...	8.1	...	...	...	10.0	...	4.0	7.3
Blood level µg/100 ml	5.9	5.6	5.4	...	...	6.4	...	...	..	5.4	...	4.8	5.6
Period IV (14 days)													
Week I													
Intake: µg/24 hr.	4964	4862	4938	...	...	4919	...	...	...	4896	...	4822	4900
Excretion: Urinary µg/24 hr.	856	707	903	...	...	792	...	...	...	939	...	641	805
Week II													
Intake: µg/24 hr.	4968	4849	4951	...	...	4941	...	...	...	4917	...	4856	4914
Excretion: Urinary µg/24 hr.	1458	1477	1388	...	...	1592	...	...	...	1522	...	1199	1439
Period V (20 days)													
Intake:													
µg/24 hr.	1087	972	1064	...	...	1054	...	...	...	1050	...	936	1027
Mg/1000 Cal.	0.50	0.49	0.57	...	...	0.49	...	...	...	0.49	...	0.67	0.54
µg/kg	23.5	13.6	23.2	...	...	20.7	...	...	...	17.7	..	13.5	18.7
Excretion:													
Urinary µg/24 hr.	254	214	147	...	...	204	...	...	...	212	...	146	196
Fasting µg/hour	16	13	9	...	...	7	...	...	..	6	...	7	10
Test dose % return	10.6	9.1	10.8	...	...	13.2	...	...	...	14.3	...	6.9	10.8
Blood level µg/100 ml	6.3	5.5	6.1	...	...	6.2	...	...	...	6.7	...	6.3	6.2

[1] Daily intakes and urinary excretions during periods I-a, I-b, II and III represent the average of 3-day collections made weekly throughout these periods with the exception of those for subject 3 in periods I-a and I-b which represent a single 3-day collection at the end of these periods.

[2] Values for the three subjects who did not participate in the first three periods are not included in this average.

stipation. At the end of the period, however, another careful examination was made and again no deficiency symptoms were observed.

The thiamine intake was increased to approximately 400 μg or 0.20 mg per 1000 Cal. in period 1-b. The daily urinary excretions increased only slightly but the blood levels and the fasting 1-hour excretions returned to approximately the same values as were found in the preliminary period. In contrast, the test dose returns were even lower than in period 1-a. They averaged only 0.7% with only two subjects returning more than 0.8%. At this time, although no deficiency symptoms were apparent, the complaints of the same four subjects (7, 8, 9 and 11) increased. In spite of the greater thiamine intake, their constipation had become more severe; in addition they complained of "difficulty in grasping ideas" and "inability to concentrate." Because they felt that their scholastic standings were being jeojardized, these four subjects withdrew from the experiment at this time. Of the five remaining, one (3) was in a highly nervous state and also found it difficult to concentrate, but continued on the experiment. The others, when questioned, reported that they had noticed no change in their state of health.

Although these symptoms are admittedly subjective, the facts (1) that these subjects were the ones who had the poorest tissue stores at the beginning of the study; (2) that their symptoms were the same as some of those reported by Elsom ('42), Williams ('40, '42, '43b), Foltz ('44) and their co-workers, and (3) that subject 3 who remained on the experiment, improved markedly on the next level of intake, lend credence to the belief that they were manifestations of a subclinical thiamine deficiency.

In period II the average daily thiamine intake was increased to slightly over 700 μg or 0.36 mg per 1000 Cal. The daily excretions showed a small increase but still averaged less than 50 μg. The fasting 1-hour excretions increased slightly and the blood levels of all rose to 6 μg or more per 100 ml. The test dose returns, however, remained low. The average re-

turn for the group was only 4.0% and only one subject (1) returned more than 5.0%. It would seem that, as judged by the usual standards, none of the intakes up to this point was adequate for this group of young women.

In period III when the intake was increased to approximately 900 μg or 0.51 mg per 1000 Cal., the daily urinary excretions averaged 107 μg, although those of three of the six subjects still were only 80 μg or less. A comparison of the average fasting 1-hour excretions, blood values and test dose returns of this period with those of period II may not be justifiable because three new subjects entered in period III. If the results of the subjects who participated in both periods II and III (subjects 1, 2 and 3) are considered, it will be seen that the fasting 1-hour excretions showed no definite trend, that the blood values were lower in every instance,[6] but that the test dose returns were all higher. The return of only one subject (1), however, was above 10% and of the three new subjects, only one had a test dose return comparable to that which she had on a freely chosen diet. These considerations would suggest that the thiamine concentration of the tissues of these subjects had increased only moderately on this level of intake; that the tissues were by no means "saturated."

That this was the case is also borne out by the results obtained in period V after 2 weeks of supplementation in period IV. The average intake in period V was 0.54 mg per 1000 Cal., approximately the same as in period III. At the end of this period the daily excretions of all the subjects were higher than they were previous to the supplementation; the fasting 1-hour excretions were higher in five and the blood levels were higher in four of the six subjects and the test dose returns were larger in all but one case (1), the average return for the group being 10.8%. According to the concept of Melnick, Field and Robinson ('39), if the tissues of these subjects had been saturated with thiamine in period III, the 2 weeks'

[6] The lower blood levels cannot be explained unless those at the end of period II were unduly high because of atmospheric conditions existing at that time which might have had a dehydrating effect.

supplementation in period IV would not have caused increased excretions at the end of period V, 2 weeks after the supplementation had been discontinued. In all but one case (subject 1) these subjects did show definitely increased excretions. It would seem, therefore, that the tissue concentrations of only one of these subjects (1) approached saturation on the highest level of intake studied. The fact that the daily urinary excretions of all the subjects were greatly increased in the last 3

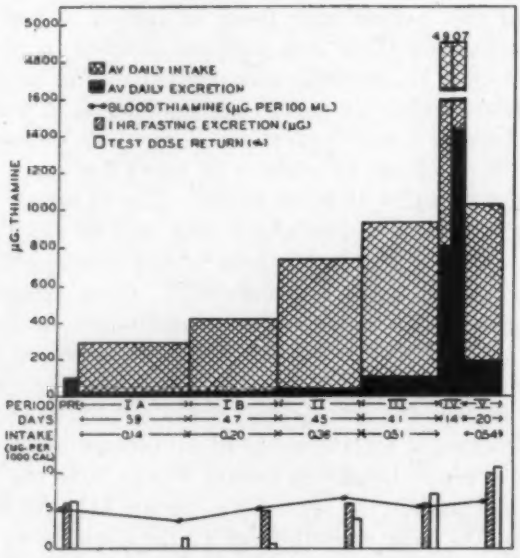

Fig. 1 Average daily excretions of thiamine, test dose returns, fasting excretions and blood thiamine at different levels of intake.

days of supplementation as compared to the first 3 days gives further support to this view and suggests that saturation had not been reached on the 0.51 mg per 1000 Cal. level even in the case of subject 1.

In interpreting these results in terms of practical allowances, it is recognized that a higher "requirement" is usually arrived at when the intakes are being progressively in-

creased from a state of depletion, than when they are being decreased from higher levels. It is possible, then, that this level of 0.5 mg per 1000 Cal. might have been sufficient to maintain tissue concentrations after they had once been increased. It should be noted, however, that although the thiamine-calorie ratios were of the order generally considered to be satisfactory, the caloric intakes and hence the total thiamine intakes were relatively low. All were less than 1 mg and two were less than 0.9 mg. For two of the largest subjects (2 and 12) whose test dose returns were only 5 and 4%, this provided only 12–13 μg per kg; for the others whose returns were somewhat higher, it amounted to 16 to 21 μg per kg. It is possible that the thiamine-calorie ratio alone is not a satisfactory basis for predicting thiamine needs, especially at low caloric intakes. These results suggest that the total daily thiamine intake of young women should probably not be less than 1 mg or 20 μg per kg of body weight regardless of the actual calories consumed. At a 2000-cal. level this would provide a thiamine-calorie ratio of 0.50. This is approximately the ratio suggested as adequate by Melnick ('42), Williams and associates ('43a), and Foltz, Barborka and Ivy ('44).

Fecal excretions of thiamine

The fecal excretions of total thiamine [7] which were determined for some of the subjects during the last week on the different levels of intake (table 2) showed no consistent relation to the intakes. Only one subject (3) showed a gradual increase in excretion as the intakes were increased and it was not always large enough to be considered significant. It will be noted, however, that three of the four subjects did have significantly higher excretions in period II when the daily intake was increased to approximately 700 μg than in period 1-b when the daily intake was in the neighborhood of 400 μg.

[7] The fecal analyses were made by Lois Buttles. The results were reported in detail in her master's thesis, "The Total Thiamine Excreted Through the Gastrointestinal Tract by Human Subjects." University of Chicago, 1945.

TABLE 2

Average daily urinary and fecal thiamine excretions on different intakes.[1]

SUBJECT	1	2	3	4	5	6	7	12
Period I-a								
Intake, μg	...	...	288	303	305	...	343	...
Excretion:								
Urine, μg	...	...	22	25	24	...	22	...
Feces, μg	...	...	267	108	351	...	211	...
Total, μg	...	...	289	133	375	...	233	...
Period I-b								
Intake, μg	366	375	402	392	372	...	...	...
Excretion:								
Urine, μg	29	16	19	29	10	...	...	...
Feces, μg	405	213	306	104	221	...	...	...
Total, μg	434	229	325	133	231	...	...	...
Period II								
Intake, μg	710	640	741	738	...	...	...	...
Excretion:								
Urine, μg	78	65	41	35	...	...	...	...
Feces, μg	459	354	318	221	...	...	...	...
Total, μg	537	419	359	256	...	...	...	...
Period III								
Intake, μg	1041	974	1046	...	...	1063	...	1010
Excretion:								
Urine, μg	116	107	97	...	...	160	...	68
Feces, μg	348	323	366	...	...	35	...	41
Total, μg	464	430	463	...	...	195	...	109
Period V								
Intake, μg	1087	972	1064	...	...	1054	...	936
Excretion:								
Urine, μg	254	214	147	...	...	204	...	146
Feces, μg	437	430	388	...	...	65	...	31
Total, μg	691	644	535	...	...	269	...	177

[1] All figures in this table are based on collections made for 3 days during the last week of each period.

There was little direct evidence of bacterial synthesis of thiamine by the intestinal bacteria as only two subjects actually excreted more thiamine than they received in their diets and this occurred in only one period in both cases. There are certain indications, however, that it did occur. The subjects who began on the low levels of thiamine and riboflavin intake (1, 2 and 3) excreted from 7 to 10 times the amount of thiamine in periods III and V as subjects 6 and 12 who entered the experiment later and had never been on the low levels. Since they were all receiving the same diet in the later periods, either some factor related to their earlier diets must have caused the increased excretions or there were pronounced individual differences. It is unfortunate that fecal collections were not made in the preliminary period, for if something in the experiment was the cause of the increased output, the effect must have taken place in period I-a, since all the subjects excreted higher amounts of fecal thiamine in that period than subjects 6 and 12 did in periods III and V.

There were many differences in the diets of period I-a as compared to periods III and V, which may have exerted an influence on the intestinal flora. The amounts of both riboflavin and thiamine were much lower in period I-a; there were more cereals and less vegetables and fruit in period I-a; the subjects consumed much larger amounts of bread in period I-a and subjects 1, 2 and 3 received, on the average, 5.8% more carbohydrate in period I-a than in periods III and V. Which, if any, of these factors operated to cause an increased synthesis of thiamine, it is difficult to say.

Niacin and pantothenic acid

Both intakes and excretions were analyzed for niacin and pantothenic acid the last week of each period with the exception of period IV, when all collections were analyzed. Although the findings were not conclusive, they are presented here because of the paucity of data concerning the intakes and excretions of these vitamins. The individual results and the averages for the group are presented in table 3.

TABLE 3

Average daily niacin and pantothenic acid excretions on different intakes.[1]

SUBJECT	PERIOD I-a				PERIOD I-b				PERIOD II				PERIOD III				PERIOD IV				PERIOD V			
	Intake	Excretion			Intake	Excretion			Intake	Excretion			Intake	Excretion			Week 1		Week 2		Intake	Excretion		
		Urine	Feces	Total		Urine	Feces	Total		Urine	Feces	Total		Urine	Feces	Total	Intake	Urinary excretion	Intake	Urinary excretion		Urine	Feces	Total
Niacin																								
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
1	12.1	1.1	..	..	10.9	1.2	0.6	1.8	12.5	1.0	0.4	1.4	16.1	0.6	0.7	1.3	23.8	1.2	23.8	1.3	15.2	1.0	0.4	1.4
2	...	..	..	..	6.9	0.9	0.6	1.5	9.7	1.1	3.0	4.1	13.3	1.3	1.1	2.4	25.0	2.0	24.7	1.3	16.0	1.2	1.5	2.7
3	9.3	0.8	0.8	1.6	9.4	0.7	1.1	1.8	11.0	0.8	1.9	2.7	14.5	0.7	1.3	2.0	22.4	0.9	22.7	0.9	13.2	0.7	1.2	1.9
4	8.7	0.9	0.8	1.7	8.6	0.9	0.9	1.8	10.6	0.8	1.4	2.2	...	..	..	..	...	..	...	..	...	..	..	..
5	8.9	0.8	1.0	1.8	7.6	0.6	1.7	2.3	9.6	0.6	..	..	...	..	..	..	...	..	...	..	...	..	..	..
6	...	..	..	..	...	..	..	..	..	..	..	..	14.7	0.6	1.5	2.1	23.3	1.0	24.0	0.8	14.4	0.8	1.4	2.2
7	11.5	1.4	0.9	2.3	...	..	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
8	14.9	1.4	..	..	13.3	1.4	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
9	10.1	1.2	..	..	8.7	1.2	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
10	...	..	..	..	...	..	..	..	..	..	..	..	13.7	0.6	..	..	22.8	0.9	23.4	0.9	14.6	0.8	..	..
11	12.8	1.0	..	..	11.9	1.4	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
12	...	..	..	..	...	..	..	..	..	..	..	..	14.0	0.6	2.6	3.2	22.6	0.8	22.6	1.0	12.6	0.7	1.3	2.0
Av.	9.6	1.0	0.9	1.9	8.7	0.9	1.0	1.9	11.0	0.9	1.7	2.6	14.5	0.8	1.4	2.2	23.3	1.1	23.5	1.0	14.3	0.9	1.2	2.1
Pantothenic acid																								
1	2.1	1.7	..	..	2.3	1.5	0.7	2.2	3.9	1.7	0.5	2.2	4.9	1.7	1.0	2.7	5.0	4.4	5.0	4.2	4.8	3.6	0.5	4.1
2	...	..	..	..	1.7	1.1	0.5	1.6	3.1	1.3	1.9	3.2	3.3	1.6	1.0	2.6	4.1	3.8	3.9	2.4	3.7	3.3	1.0	4.3
3	2.1	1.8	0.7	2.5	2.7	1.7	0.9	2.6	3.4	2.0	1.3	3.3	4.2	2.6	1.6	4.2	4.8	3.8	5.0	5.0	4.6	4.1	1.6	5.7
4	1.7	1.6	0.6	2.2	2.1	1.7	0.7	2.4	3.7	2.5	1.5	4.0	..	..	..	..	..	..	..	..	..	..	..	..
5	2.0	1.7	1.1	2.8	1.9	1.8	1.4	3.2	3.2	2.2	..	..	...	..	..	..	...	..	...	..	...	..	..	..
6	...	..	..	..	...	..	..	..	..	..	..	..	4.7	4.6	2.1	6.7	4.8	4.5	5.2	5.4	4.8	4.0	1.3	5.3
7	2.6	1.7	0.8	2.5	...	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
8	3.3	1.6	..	..	3.0	1.7	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
9	2.3	1.8	..	..	2.4	1.5	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
10	...	..	..	..	...	..	..	..	..	..	..	..	4.7	2.3	..	..	4.5	3.9	4.8	3.3	4.7	2.8	..	..
11	2.2	1.4	..	..	2.5	1.6	..	..	..	..	..	..	...	..	..	..	...	..	...	..	...	..	..	..
12	...	..	..	..	...	..	..	..	..	..	..	..	4.3	2.2	2.1	4.3	4.5	3.2	4.6	3.9	4.0	2.9	1.1	4.0
Av.	2.1	1.7	0.8	2.5	2.1	1.6	0.8	2.4	3.5	1.9	1.3	3.2	4.3	2.5	1.6	4.1	4.6	3.9	4.8	4.0	4.4	3.6	1.1	4.7

[1] All figures in this table are based on collections made for 3 days during the last week of each period.

The average daily niacin intakes of the subjects ranged from 8.7 to 14.5 mg previous to supplementation, and were 23.4 mg during this period. In spite of this wide range in intake, the average urinary excretions remained relatively constant in the neighborhood of 1.0 mg. This represented approximately 10% of the lower and 5% of the higher levels of intake. The urinary excretion of approximately 1.0 mg is in agreement with the findings of Sarett, Huff and Perlzweig ('42) and Goldsmith ('42, '44), on normal individuals who were consuming different types of diets. Briggs and co-workers ('45) also have reported continued urinary excretions of approximately this magnitude by a subject whose daily niacin intake was 3 mg or less for a period of 42 weeks. This tendency toward constant niacin excretions is unlike that of both riboflavin and thiamine, which respond promptly to changes in intake.

The average fecal excretions of niacin on the lowest levels of intake in periods I-a and I-b were approximately the same as the urinary excretions but were somewhat higher on the higher intakes. However, they were never more than 15% of the intake. For the most part both the urinary and fecal excretions of the different subjects did not vary greatly from the average.

Both the intakes and excretions of pantothenic acid found in this study were of the same order of magnitude as those found by other workers on individuals consuming self chosen diets. The low levels of intake of these subjects correspond closely to the average intake of 2.4 mg found in a group of twenty-four women with low incomes; the higher levels to that of 4.8 mg in a group of twelve women with moderate incomes (Winters and Leslie, '43, '44). The urinary excretions on the higher levels of intake approximated the average amounts of 3.8, 3.2 and 3.4 mg reported on groups of normal individuals by Pelczar and Porter ('41), Pearson ('41) and Wright and Wright ('42).

The pantothenic acid excretions present a decidedly different picture from those of niacin. At every level of intake

from 2.1 to 4.7 mg, the average total excretions consistently approximated the intakes (table 3). When individual values are considered, the tendency was for the excretions to exceed the intakes by small amounts. From 54 to 84% of the excreted pantothenic acid was found in the urine and from 25 to 38% in the feces. This agrees with the findings of Gardner, Neal, Peterson and Parsons ('43) although their subjects excreted a slightly higher proportion in the urine and a smaller one in the feces.

These results are also in harmony with the suggestion of the Wisconsin group that either no pantothenic acid is destroyed in the body or that intestinal synthesis compensates for the destruction in the tissues. That synthesis could adjust to different intakes as precisely as was the case in this study, however, seems rather doubtful. In view of the findings here presented the former theory seems more plausible. In any case, the validity of a daily allowance of the 10 mg suggested by Williams ('42) is questionable.

SUMMARY

Daily urinary and fecal thiamine excretions, test dose returns, blood thiamines and 1-hour fasting thiamine excretions of twelve women were determined on various levels of intake over a period of 8 months. Intakes of 0.14, 0.20 and 0.36 mg per 1000 Cal. were judged to be inadequate on the basis of urinary excretions and test dose returns. On an intake of 0.51 mg per 1000 Cal. the excretions increased somewhat but still remained low. That the tissues were not saturated at this level was further indicated by the facts that (1) during subsequent supplementation the excretions were higher the last 3 days than the first 3; and (2) in the final period on an intake similar to that previous to supplementation, both daily urinary excretions and test dose returns showed increases. It is suggested that the total daily thiamine intake should probably not be less than 1 mg or 20 μg per kg of body weight.

Fecal thiamine excretions for different individuals were relatively constant throughout the study. Average daily uri-

nary and fecal excretions of niacin also remained relatively constant at approximately 1.0 mg each, regardless of the levels of intake. Total excretions of pantothenic acid closely approximated the intakes at each level, by far the larger proportion being excreted in the urine.

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THE ISOLEUCINE, LEUCINE AND VALINE REQUIREMENTS OF THE CHICK

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The qualitative requirements of the chick for leucine, isoleucine and valine have been reported previously (Grau and Almquist, '44; Hegsted, '44; Almquist and Grau, '44). In these studies it was found that the omission of any of these amino acids from the diet caused an immediate loss in weight and eventual death. The present report is concerned with the effects on growth of different levels of these amino acids in the diet and with the effectiveness of their optical isomers in promoting growth.

METHODS

White Leghorn chicks were fed a commercial-type mash until they were 10 days old. From the tenth to the fourteenth day they were placed on a conditioning diet with the following composition (in grams): cellulose [1] 5, calcium gluconate 8, mineral mixture 3.8, crude soybean oil 5, sardine oil (400 D–3000 A per gm) 0.25, natural mixed tocopherols [2] 0.05, choline chloride [3] 0.2, inositol 0.1, cholic acid 0.1, 2-methyl-1, 4-naphthohydroquinone diacetate 0.001, thiamine 0.001, riboflavin 0.001, pyridoxine 0.001, nicotinic acid 0.003, calcium (d) panto-

[1] Cellu flour.

[2] Natural mixed tocopherols (15%), Distillation Products, Inc.

[3] Choline chloride was provided by Lederle Laboratories, Inc., through the courtesy of Dr. T. H. Jukes.

thenate 0.003, biotin [4] 0.00001, folic acid [5] 0.0001, casein 25, gelatin 7, and glucose [6] to make 100 gm. The mineral mixture contributed the following materials in grams to each 100 gm of diet: tricalcium phosphate 2.0, dipotassium phosphate 0.5, potassium chloride 0.3, manganese 0.01, silicon 0.046, magnesium 0.048, aluminum 0.008, iron 0.014, copper 0.001, zinc 0.001, iodine, 0.0008, and cobalt 0.0005 gm.

The chicks were weighed daily until they were 2 weeks old, when groups of four closely selected chicks were fed the experimental diets. The selection was made so that all groups had approximately equal weight distributions and average weights. Only chicks which grew uniformly during the second week were used. The range of weights of the chicks chosen was between 84 and 100 gm for the first experiment and between 72 and 99 gm for the second. The chicks were housed in electrically heated battery brooders with wire floors. Feed and water were supplied ad libitum. The basal experimental diet was the same as the conditioning diet except for the amino acid source, which in the experimental diet consisted of a mixture of commercial amino acids. The amino acid mixtures used as positive controls in the two experiments are shown in table 1. Sodium bicarbonate was added to neutralize the hydrochlorides of the basic amino acids and to provide sodium chloride, which was omitted from the mineral mixture used. As the amount of an amino acid in the diet was decreased, an equal weight of glucose was added to make up the difference.

The amino acids used were commercial products, except for the optical isomers of isoleucine and valine, which were prepared according to the methods of Locquin ('07) and Fischer ('06), respectively. The optical rotations of these preparations are given in table 2.

[4] Biotin was furnished by Merck and Co., Inc., through the courtesy of Dr. J. C. Keresteszy.

[5] Folic acid was provided by Lederle Laboratories, Inc., through the courtesy of Dr. E. L. R. Stokstad.

[6] Cerelose.

TABLE 1

Mixtures of amino acids used in the positive control diets.

AMINO ACID	FORM	LEVEL AS FED		AMINO ACID	FORM	LEVEL AS FED	
		Expt. 1	Expt. 2			Expt. 1	Expt. 2
		%	%			%	%
Alanine	dl	1.5	1.5	Lysine	l(+)·HCl·H_2O	1.4	1.4
Arginine	l(+)·HCl	1.4	1.4	Methionine	dl	0.6	0.6
Aspartic acid	dl	1.0	..	Norleucine	dl	0.2	..
Cystine	l(−)	0.5	0.5	Phenylalanine	dl	1.5	1.0
Glutamic acid	l(+)	5.0	7.0	Proline	l(−)	2.0	..
Glycine	.....	1.8	1.8	Threonine	dl	3.0	1.3
Histidine	l(+)·HCl·H_2O	0.8	0.8	Tryptophane	l(−)	0.3	..
Hydroxyproline	l(−)	0.2	..	Tryptophane	dl	..	0.6
Isoleucine	dl	2.0	1.0	Tyrosine	l(−)	2.0	2.0
Leucine	l(−)	2.0	1.5	Valine	dl	2.0	1.5
				($NaHCO_3$)		1.5	1.5

TABLE 2

Specific rotations of the isomers of isoleucine and valine in 20% HCl at 20°C.

AMINO ACID	FOUND	RECORDED IN REFERENCE	REFERENCE
l(+)-Isoleucine	+ 39.1°	+ 40.61	Locquin ('07)
d(−)-Isoleucine	− 39.0°	− 40.86	Locquin ('07)
l(+)-Valine	+ 28.6°	+ 28.7	Fischer ('06)
d(−)-Valine	− 28.2°	− 28.4	Fischer ('06)

The chicks and feed were weighed daily during the experiments, which lasted 10 days. Individual growth curves were plotted, and from these a smooth curve was drawn for each chick. The initial and final corrected weights were obtained from the curve, and the per cent gain per day was calculated according to the formula:

$$\% \text{ gain per day} = \frac{\text{ave. gain} \times 100}{\text{ave. wt.} \times \text{no. of days}}$$

In order to obtain the ratio of gain to feed, the sum of the corrected weight gains was divided by the total feed consumed.

RESULTS AND DISCUSSION

The growth rates with the various diets as well as the range of the individual rates are given in table 3.

TABLE 3

The effects of different levels of isoleucine, leucine and valine on the rate and efficiency of gain. There were four chicks in each group; the experiment lasted 10 days.

AMINO ACID	LEVEL OF AMINO ACID IN DIET	OPTICAL FORM	PER CENT GAIN PER DAY		GAIN PER GM FEED CONSUMED
			mean	range	
	%				gm
	0	..	−1.3	−1.9, −0.5	−0.23
		d(−)	−0.7	−1.9, +0.3	−0.12
	0.45[1]	dl	+1.3	+0.1, 2.0	+0.15
		l(+)	4.1	3.3, 5.2	0.35
Isoleucine	0.5	dl	1.2	0.0, 2.1	0.17
	1.0	dl	4.0	3.3, 4.3	0.42
	1.5	dl	3.6	3.1, 4.0	0.40
	2.0	dl	3.3	2.2, 4.7	0.39
	0	..	−2.7	−3.2, −1.6	−0.53
	0.5	l(−)	−1.1	−1.4, −1.0	−0.24
	1.0	l(−)	+1.8	+1.5, 2.2	+0.24
Leucine		dl	2.0	1.3, 2.6	0.25
	1.5	l(−)	3.4	3.2, 3.5	0.41
	2.0	l(−)	3.3	2.2, 4.7	0.39
		dl	3.2	2.9, 3.9	0.45
	0	..	−2.8	−3.1, −2.5	−0.64
	0.5	dl	−1.3	−1.6, −0.8	−0.32
		d(−)	−0.3	−0.7, +0.3	−0.05
	0.7[1]	dl	+1.6	+1.4, 1.8	+0.16
Valine		l(+)	3.8	2.0, 4.7	0.35
	1.0	dl	1.3	0.9, 1.8	0.18
	1.5	dl	3.4	1.9, 5.1	0.42
	2.0	dl	3.3	2.2, 4.7	0.39

[1] Results obtained from the second experiment.

Isoleucine

As the level of dl-isoleucine in the diet was increased the growth rate increased rapidly until a rate of 4% per day was obtained when 1% of isoleucine was fed, after which the rate decreased slightly when the isoleucine content was increased to 2%. In the second experiment the growth rates obtained with equal levels (0.45%) of dl-, d(—)-, and l(+)- isoleucine were compared. The data show (table 3) that d(—)- isoleucine cannot be utilized for the promotion of growth in the young chick and that the l(+) form is twice as effective as the dl form in this regard. The level chosen allowed the maximum differences in growth between the three forms. These data indicate that the requirement for l(+) isoleucine is approximately 0.5% of the diet since the maximum growth was obtained at 1% dl-isoleucine.

Leucine

In the absence of leucine, the average loss per day was 2.7%. As the leucine content of the diet was increased, growth increased until, at the 1.5% level of l(—) leucine, the growth was 3.4%. From the growth rates as shown in table 3, it is seen that the l(—)-leucine requirement is approximately 1.5% of the diet. The growth rates with 1% and 2% of dl-leucine appear to be equivalent to those obtained with equal levels of l(—)-leucine. (If only l(—)-leucine were utilizable for growth, the expected gain with 1% dl-leucine would be about —1%, while 2% dl-leucine would allow a growth rate of only 2%).

Valine

Growth at the 1.5% level of dl-valine was as good as that obtained with 2% of the dl-form. When the optical isomers were fed, an average gain of 3.8% was obtained for the 10-day period with 0.7% l(+)-valine, a loss of weight (0.3% per day) occurred with 0.7% of d(—)-valine, and an intermediate value (1.6%) was obtained with 0.7% dl-valine. From these results, the requirement is estimated to be 0.7% of the natural form.

The chick is able to utilize only the natural forms of isoleucine and valine for growth, and in this respect is the same as the mouse (Bauer and Berg, '43) and the rat (Rose, '38). The metabolism of leucine in the chick, on the other hand, appears to be different from that in the rat and the mouse, since only the natural form is utilized for growth in the latter animals. This is the only instance which has been found thus far in which the requirements of the chick are less specific than those of other animals.

SUMMARY

Use of a diet containing mixtures of amino acids has shown the requirements of the chick for best growth to be: l(+)-isoleucine, approximately 0.5% of the diet; l(—)-leucine, 1.5%; and l(+)-valine, 0.7%. d(—)-Isoleucine and d(—)-valine are not utilized for growth, but dl-leucine appears to be as effective as l(—)-leucine in promoting growth.

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THE PANTOTHENIC ACID CONTENT OF THE BLOOD AND TISSUES OF THE CHICKEN AS INFLUENCED BY THE LEVEL IN THE DIET

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The effect of the amount of the various B vitamins in the diet on the amounts in the tissues of animals normally used for human food has been studied to only a limited extent. The thiamine content of pork tissue has been shown by several workers (Hughes, '41; Miller et al., '43; and Heinemann et al., '46) to be related to the amount ingested.

While information is available on the amounts of various B vitamins in the egg, very little is known about the influence of the amount of any of the B vitamins in the diet on the level in the tissues and blood of the chicken. Snell, Pennington and Williams ('40) fed four chicks on a diet furnishing an adequate amount of pantothenic acid and four on a diet containing an insufficient amount. They found that the pantothenic acid content of the tissues of 26-day-old chicks was influenced by the amount of pantothenic acid in the diet. Pearson, Melass and Sherwood ('45) have shown that the level of pantothenic acid in the diet determines to a large extent the concentration in the egg, but that irrespective of the amount at the beginning of incubation, the amount in the egg changes very little during embryonic development. The pantothenic acid content of the blood of various mammalia and its distribution between plasma and cells has been reported by Pearson ('41). No comparable information is available for the chicken.

This investigation was carried out to obtain information on the influence of the amount of pantothenic acid in the diet of the chicken on the levels in the tissues and the blood.

EXPERIMENTAL

Methods

The dietary treatment of the chickens was essentially the same as described in a previous publication (Pearson et al., '45). The hens used in these experiments were chiefly White Leghorns. They had been fed the experimental diets, which differed only in respect to the amounts of pantothenic acid, for a period ranging from 12 to 24 months. The two levels of pantothenate in the diets were 385 μg and 1575 μg per 100 gm of feed.

The blood for analyses was drawn directly from the heart into an oxalated tube. Pantothenic acid assays were made on the whole blood, the plasma and cells. The plasma and cells were separated by centrifuging the whole blood for 50 minutes at approximately 2500 r.p.m.

Pantothenic acid assays were made on the liver, the breast tissue and the leg muscle. Pantothenic acid was determined by the microbiological method of Neal and Strong ('43) after liberation of the vitamin by autoclaving and enzymatic treatment essentially as described for newly hatched chicks (Pearson et al., '45).

Blood studies

The whole blood of chickens on the 1575 μg level of pantothenic acid, which may be considered approximately adequate to meet the dietary requirements, contained an average of 43.6 μg of pantothenic acid per 100 ml. This is of the same order as the values for mammalia (Pearson, '41) which ranged from 19.4 μg for human blood to 71.7 μg per 100 ml of blood for rabbits. In these same studies horse blood was found to contain an average of 44.8 μg of pantothenic acid per 100 ml.

The level of pantothenic acid in the whole blood, the plasma and the cells (table 1) is definitely influenced by the amount of

pantothenic acid in the diet. The pantothenic acid levels for the hens on the 1575 μg level were, for whole blood 43.6 μg, for plasma 51.6 μg, and for cells 21.9 μg per 100 ml, respectively, as compared to 20.0 μg, 20.9 μg, and 13.0 μg per 100 ml, respectively, for the hens on the 385 μg level of pantothenic acid. The percentage difference between the values of the two groups are 118 for whole blood, 147 for plasma and 40.6 for the cells. The mean differences between the pantothenic acid values for the whole blood, plasma and cells of the hens on the two different dietary levels are each highly significant according to Fisher's *t* test.

TABLE 1

The pantothenic acid content of chicken blood as influenced by amount in the diet.

PANTOTHENIC ACID IN THE DIET	NO. OF BIRDS	CELLS		BLOOD		PLASMA		CELLS	
		Mean	S.D.[1]	Mean	S.D.	Mean	S.D.	Mean	S.D.
		Vol. %		*μg/100 ml*		*μg/100 ml*		*μg/100 ml*	
385 μg/100 gm	17	26.8	3.7	20.0	6.9	20.9	8.5	13.0	2.6
1575 μg/100 gm	17	27.3	3.4	43.6	9.7	51.6	9.5	21.9	6.2

[1] Standard deviation.

The data on the effect of the level of dietary pantothenic acid on the level in the blood of chickens are in accord with the observation (Silber, '44) that dogs depleted of pantothenic acid have less pantothenic acid in their blood than the blood of dogs on stock diets.

There was no significant difference in the volume per cent of cells in the blood of the hens fed the high and low levels of pantothenic acid.

On the basis of the level of pantothenic acid found in the plasma and cells, the calculated amount for whole blood, as shown in table 2, agrees fairly well with the observed or determined value for whole blood (table 1). The percentage distribution of pantothenic acid between the plasma and cells based on the values per 100 ml each of cells and plasma has been calculated and is presented together with the per cent cells in table 2. The table shows that 86.2% of the pantothenic

acid of the blood of the hens fed the 1575 μg level occurs in the plasma, whereas in mammalia the per cent of pantothenic acid that occurs in the plasma ranges from 44.3 to 62.6 (Pearson, '41).

TABLE 2

Calculated amount of pantothenic acid in whole blood and its distribution between plasma and cells.

PANTOTHENIC ACID IN DIET	CALCULATED AMOUNT IN WHOLE BLOOD [1]	VARIATION FROM DETERMINED VALUE FOR WHOLE BLOOD	PER CENT OF TOTAL P.A. IN WHOLE BLOOD OCCURRING IN:	
			Plasma	Cells
	μg/100 ml	*μg/100 ml*		
385 μg/100 gm	18.7	— 1.4	81.7	18.3
1575 μg/100 gm	43.5	— 0.1	86.2	13.8

[1] Calculated amount in whole blood — per cent cells times pantothenic acid per 100 ml of cells plus per cent plasma times pantothenic acid per 100 ml of plasma.

Tissue studies

The influence of the level of pantothenic acid in the diet on the content in tissues is shown in table 3. The liver of the hens on the low pantothenic acid intake contained an average of 40.7 μg of pantothenic acid per gram of fresh tissue as compared with 44.9 μg for the livers of the hens on the high intake.

TABLE 3

The effect of the amount of pantothenic acid in the diet of the chicken on the amount in the tissues.

PANTOTHENIC ACID IN DIET	NO. OF BIRDS	LIVER		LEG MUSCLE		BREAST TISSUE	
		Mean	S.D.[1]	Mean	S.D.	Mean	S.D.
		μg/gm		*μg/gm*		*μg/gm*	
385 μg/100 gm	15	40.7	7.8	8.4	1.9	6.5	1.2
1575 μg/100 gm	15	44.9	6.9	17.2	2.8	11.3	3.2

[1] Standard deviation.

According to Fisher's *t* value there is no significant difference in these two values. The similar pantothenic acid values for the livers of chickens on different dietary levels is in contrast to the findings of Silber ('44) with dogs. This investigator

reported that the livers from dogs depleted of pantothenic acid contained significantly less pantothenic acid than the livers of dogs fed a stock diet. On the other hand, Heinemann et al. ('46), working with pigs, fed different levels of riboflavin without influencing the riboflavin content of the liver. Furthermore, the subsequent feeding of a diet containing approximately 3950 μg of pantothenic acid per 100 gm did not further increase the amount in the liver of chickens. The data suggest a species difference between the dog and the chicken with respect to the influence of the amount of pantothenic acid in the diet on the concentration in the liver.

The pantothenic acid was determined on livers from eight chickens 9 weeks of age that had been fed a stock diet from the time of hatching. The average pantothenic acid value for the livers from the chickens 9 weeks of age was 43.1 μg per gm of fresh tissue, which is not significantly different from the values reported here for older chickens. From this it appears that age is not a factor in the amount of pantothenic acid in the liver of chickens.

The level of pantothenic acid in the leg muscle is higher than in the breast tissue. The difference between the level of pantothenic acid in the leg muscle and the breast tissue is highly significant for both the high and low level of pantothenic acid intake.

The leg muscle of the chickens on the high pantothenic acid intake contained an average of 17.2 μg per gm of fresh tissue as compared with an average of 8.4 μg for the group on the low intake. The breast tissue of the chickens on the high pantothenic acid level contained an average of 11.3 μg per gm as compared with 6.5 μg of pantothenic acid for the chickens on the low level. The differences between the values for the two levels of pantothenic acid are highly significant for both the leg muscle and breast tissue. Similarly the amount of pantothenic acid in the diet of dogs was found by Silver ('44) to influence the amount of pantothenic acid in the leg muscle.

The rate at which depleted tissues replenish their store of pantothenic acid was studied by changing hens from the 385

μg level of pantothenic acid to the 1575 μg level. One group of four chickens was killed at the end of 14 days and a second group after 28 days, and the tissues assayed for pantothenic acid. At the end of the 14-day period the average pantothenic acid content of the leg muscle was 17.9 μg per gm, which is essentially the same as for the chickens that had continuously been on the high intake. However, the average value for the breast tissue at the end of 14 days was only 8.8 μg as compared with 11.3 μg of pantothenic acid per gm. These data indicate that the rate of storage of pantothenic acid is more rapid in the leg muscle than in the breast tissue of the chicken. After 28 days on the high intake the pantothenic acid content of the breast tissue had increased to the same order as for birds that had continuously been on the high intake and there was no significant change from the 14-day-period in the concentration of pantothenic acid in the leg muscle.

In order to determine if the pantothenic acid content of breast tissue and leg muscle could be increased above the level for the 1575 μg level of intake, the amount of pantothenic acid in the diet of some of the chickens from this group was increased to approximately 3950 μg per 100 gm of feed. Four weeks later the levels of pantothenic acid in the tissues of the chickens were not significantly different from the average for the birds kept continuously on the 1575 μg level.

SUMMARY

Data are presented on the effect of the amount of pantothenic acid in the diet of chickens on the level in the blood and tissues.

On an adequate intake of pantothenic acid (1575 μg per 100 gm of feed) the average level in whole chicken blood was 43.6 μg per 100 ml and for plasma 51.6 μg. These values are 118 and 147% higher than the respective values for the blood and plasma of chickens on an intake of about 385 μg of pantothenic acid per 100 gm of feed. In whole chicken blood an average of 86.2% of the pantothenic acid occurs in the plasma, which is

somewhat higher than the corresponding figure for the blood of mammalia.

The amount of pantothenic acid ingested had no significant effect on the amount of pantothenic acid in the liver. On a diet containing approximately 1575 μg of pantothenic acid per 100 gm of feed the average pantothenic acid value for liver was 44.9 μg, for leg muscle 17.2 μg and for breast tissue 11.3 μg per gm of fresh tissue. The level of pantothenic acid in the leg muscle and breast tissue was significantly influenced by the amount ingested. The addition of pantothenic acid to the diet of chickens that had been on a low intake resulted in a relatively rapid deposition of pantothenic acid in the leg muscle and breast tissue so that the amount in these tissues reach the normal level for the amount ingested within a period of 2 to 4 weeks.

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THE EFFECTS OF A HIGH FAT DIET IN A TEMPERATE ENVIRONMENT [1]

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TWO FIGURES

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The belief that the eating of meat confers strength upon the eater is prominent in the folklore of many peoples. The evidence gathered by explorers and anthropologists during the last 100 years has proved conclusively that some very vigorous groups, for example the Eskimos, live exclusively upon meat. Furthermore some explorers themselves have lived entirely by hunting and one explorer, Stefanssen, has thrived upon a meat diet under controlled experimental conditions in a New York hospital (Stefanssen, '35–'36). There is no indication that such a diet decreases vigor. In those areas where food is obtained entirely from the animal carcass, the fatty tissues seem to be particularly prized. Indeed there is evidence to suggest that fat is essential and that a diet of lean meat alone causes marked illness in a very few days (Stefanssen, '35–'36). When both lean meat and fatty tissues are available in unlimited amounts, the natural tendency seems to be to eat enough fat so that most of the calories are derived from this

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source. Stefanssen ('44) believes that as much as 80% of the calories may come from fat.

In view of these observations which have extended over many years and many cultures, it is extraordinary to read of the results of recent experiments upon diets consisting exclusively of protein and fat (Kark, Johnson and Lewis, '45) in which it was clearly shown that the subjects upon these diets were not only incapable of hard work but even appeared to be no better off than if they had gone without food altogether. This sharp discrepancy between well established facts and recent experiments has puzzled us greatly. The most obvious difference between the two sets of observations is that the diets in the earlier work consisted of meat and fat eaten either raw or after ordinary cooking, while in the work of Kark, Johnson and Lewis ('45) pemmican was used. The pemmican was prepared by dehydrating cooked lean meat, grinding it up and then mixing it with melted fat. There is nothing in this process which would be expected to change the nutritional properties of the food (except the flavor) and yet there appeared to be a very great change in these properties. Could it be the dehydration? This seemed unlikely as there was no limitation of the water intake and no reason to suppose the protein was not rehydrated in the digestive tract. The subjects in Kark's experiments were only on the diet for 3 days. Was this too short a time to accustom them to the diet? To answer these and other questions we carried out a short preliminary study with twelve men on a field trip.

METHODS

A field trial was held for 3 weeks on a small island, Pasque, near Woods Hole, Buzzards Bay, Mass. The temperature ranged from 48°F. to 70°F., the weather being rainy and misty most of the time. The subjects, including three civilians and nine enlisted men, were laboratory workers, performing the qualitative and quantitative biochemical analyses essential to the study. The group was divided so that eight men acted as subjects and four men as controls. The controls ate a good

packaged ration supplemented with bread, butter, jam, coffee, tea, and extra evaporated milk and sugar, and their daily intake of nutrients was, in all respects, at least as high as the standard recommended by the National Research Council. Their average protein intake was 105 gm per day, and fat accounted for 30% of their caloric intake. The experimental subjects ate pemmican, made by adding 50% of fat to 50% of ground dried leaf beef, with 1% salt added. On such a diet, 71% of the calories are derived from fat, 27% of the calories from protein, and 2% from carbohydrate. They had the choice of either tea or coffee without sugar or milk, and as much water as they wanted to drink. There was an initial period of 5 days on a normal diet, followed by 9 days' experimental period on pemmican and then a return to a normal diet for 4 days. Biochemical tests were made during all three periods.

On the first day of the diet, the subjects were each given a can of pemmican that weighed approximately 460 gm, containing 7 cal. per gm. They were cautioned to eat only 100 to 200 gm the first day or so and then only when really hungry. The reason for this warning was previous experience in which many subjects on changing from a normal diet to a high fat diet became nauseated and vomited during the first 2 days. A careful check was kept on the daily food consumption of each subject.

Biochemical and physiological measurements were made periodically. Body weight was measured daily. Fluid balance was calculated daily from body weight, fluid intake and fluid excretion. Physical fitness was measured by the "pack test" (Darling et al., '44; Johnson, Brouha and Darling, '42). Basal metabolic rate was measured by a Douglas bag method. Biochemical measurements and methods included: serum protein, Phillips et al. ('43); blood glucose, Folin and Malmros ('29); serum and urine chloride, Keys ('37); serum non-protein nitrogen, Daly ('33); serum cholesterol, Bloor ('26); serum and urinary ascorbic acid, Farmer and Abt ('36); urinary nitrogen, Ma and Zuazaga ('42); urinary riboflavin, thiamine and N^1-methylnicotinamide, Johnson, Sargent, Robinson and

Consolazio ('45); urinary ketone bodies, qualitative, Rothera (see Hawk and Bergeim, '37); bromsulfalein test of liver function, Rosenthal and White ('25); phenolsulfonphthalein test of kidney function; oral glucose tolerance test, Exton and Rose ('34); insulin tolerance test. Laboratory procedures were conducted by means of our portable field laboratory, Johnson ('45).

RESULTS

For the first day or so the subjects ate the pemmican warmed slightly, but this method was given up as the dehydrated meat had to be chewed for quite a long time, which made one nauseated. On the third day, one of the subjects found that after addition of water and boiling the mixture for ½ to 1 hour, it

TABLE 1

Daily intake of pemmican in grams.

SUBJECT	DAY OF DIET									TOTAL
	1	2	3	4	5	6	7	8	9	
M.C.	139	135	191	172	55	89	126	53	138	1098
F.C.	190	183	398	238	230	231	90	143	67	1770
W.F.	140	208	181	195	90	66	162	220	73	1335
P.K.	113	160	148	191	100	50	31	4	0	787
A.R.	81	61	49	39	0	0	0	0	0	230
G.S.	121	374	245	670	635	467	662	510	408	4093
J.S.	138	184	177	321	241	190	130	49	64	1494
R.W.	162	211	155	128	120	10	0	0	0	786
Ave.	136	190	193	244	184	138	150	122	94	1449

made a fair stew. This seemed to be the best way to eat the pemmican, for the amount consumed kept increasing daily up to the fourth day, to 244 gm per person (table 1). After the fourth day the amount decreased gradually, so that on the ninth and last day the average consumption was 94 gm (660 cal.). One subject, A. R., became nauseated on the first day and as a result ate only 230 gm during the whole experimental period. He felt that he would rather starve the last 5 days than run the risk of vomiting. Another subject, G.S., was quite the opposite. He ate about 200 gm per day for the first

3 days, and then ate from 400 to 674 gm daily (2800 to 4720 cal.) the remaining 6 days. For the last 3 days he ate from 50 to 150 gm more than the rest of the group put together. At the end, no subject had begun to enjoy the taste of the food nor to feel that he could live on it.

During the first few days morale was fair but deteriorated noticeably until the subjects became listless, disheartened and difficult to rouse to do any physical work. They preferred to avoid strenuous exercise, particularly if it was prolonged, but did quite well on the physical fitness test (lasting only 5 minutes), which everyone was compelled to do on every third day.

Physical fitness in the subjects, as measured by the "pack test," showed an increase in score from 65 to 74, during the first 2 days and then leveled off until the end of the diet. After 3 days on a normal diet, the score increased from 74 to 83. The controls, on the other hand, increased gradually from a score of 46 before the diet to 73, 3 days after the diet. The improvement during the first few days of the experiment is explained largely by the fact that most of the men were not in training at the beginning of the experiment. The constancy of the scores during the dietary period is misleading because the men were 5.9 kg lighter at the end of the period and no extra weights were added to the packs to compensate for their weight loss. Such a decrease in work done should be accompanied by a definite increase in score, whereas no increase in score was found. The controls improved continuouly during the dietary period, which suggested that the control diet was definitely better.

Body weight showed striking changes, the experimental subjects averaging 5.9 kg loss in weight as compared with the 1.0 kg lost by the controls (table 2). In 3 days of normal diet the experimental subjects regained 3.6 kg. Part of these changes were on a caloric basis because of the average daily caloric deficit of about 1500. Part was also due to change in water balance. In the subjects during the first 3 days on the diet more fluid was put out in the urine than was taken in by mouth; in the controls the intake always exceeded the urinary output

TABLE 2

Changes in body weight during and after pemmican.

SUBJECTS RECEIVING PEMMICAN	BEFORE	DAYS DURING				DAYS AFTER	
	1	2	4	5	7	9	1	3
M.C.	74.8	72.1	71.6	70.9	70.1	69.1	70.5	72.9
F.C.	95.0	92.1	90.6	90.5	89.5	87.7	90.3	90.8
W.F.	73.4	70.0	69.1	68.2	67.5	66.4	68.0	68.7
P.K.	75.0	72.7	72.2	71.3	70.0	69.0	71.9	72.9
A.R.	89.8	87.2	85.5	85.0	83.8	82.5	84.4	85.6
G.S.	65.9	62.8	64.2	63.1	63.7	62.9	64.5	66.3
J.S.	74.0	70.7	69.9	69.6	68.7	68.0	71.1	72.9
R.W.	66.7	65.4	64.2	64.2	61.9	60.4	62.5	65.0
Ave.	76.7	74.1	73.4	73.0	71.9	70.8	72.9	74.4
Controls								
J.E.	68.2	66.9		66.5		66.2	67.5	67.8
F.M.	79.7	79.6		79.7		79.2	79.4	79.2
J.P.	69.0	68.2		68.7		68.3	68.5	68.6
S.T.	64.3	65.0		64.6		64.1	64.2	64.8
Ave.	70.4	69.9		69.6		69.4	69.8	70.1

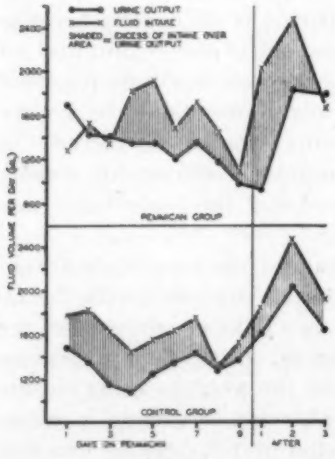

Figure 1

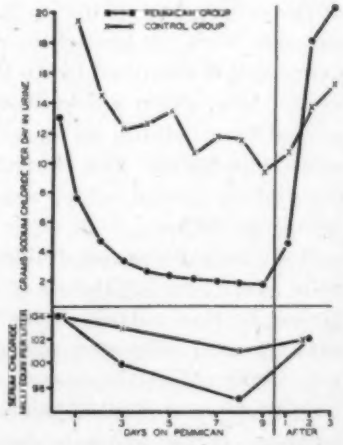

Figure 2

Fig. 1 Daily fluid intake and urine output.
Fig. 2 Serum and urine chlorides.

(fig. 1). At the same time the urinary excretion of chloride (fig. 2) and its serum level were dropping steadily in the subjects but not in the controls. For 2 days after resumption of normal diet, there was a large retention of water by the subjects with increasing serum chloride and urinary excretion of chloride.

TABLE 3

Measurements showing no significant difference between pemmican group and controls.

MEASUREMENTS AND UNITS	SUBJECTS	PERIOD OF TRIAL			
		Before 1 day	Experimental ration 4 days	9 days	After 3 days
Serum N.P.N. mg/100 ml	Pemmican	35	40	32	28
	Controls	35	28	30	27
Serum cholesterol mg/100 ml	Pemmican	190		205	175
	Controls	165		210	195
Serum ascorbic acid mg/100 ml	Pemmican	0.6	0.4	0.5	0.4
	Controls	0.3	0.2	0.4	0.4
Serum protein gm/100 ml	Pemmican	6.7	6.8	6.7	6.3
	Controls	6.5	6.6	6.2	6.5
Urine ascorbic acid mg/hour	Pemmican	0.8	0.6	0.2	0.5
	Controls	0.5	0.5	0.1	0.3
Urine thiamine μg/hour	Pemmican	11	7	7	6
	Controls	8	6	6	6
Urine riboflavin μg/hour	Pemmican	76	41	32	53
	Controls	63	46	31	42
Fasting blood glucose mg/100 ml	Pemmican	101	112	103	107
	Controls	100		107	112
Basal metabolic rate % of normal	Pemmican	+6	+9		0
	Controls	+5	+6		0
P.S.P. kidney function % excreted in 2 hrs.	Pemmican	64	69		61
	Controls	68	66		73

Of the many other measurements made, some, discussed later, showed differences between the experimental subjects and the controls, and the rest did not. The latter are listed in table 3 and included: serum NPN, serum cholesterol, serum

ascorbic acid, serum protein, fasting blood sugar, BMR, kidney function tests, urinary ascorbic acid, thiamine and riboflavin. Significant differences were found in urinary ketone bodies, nitrogen, N^1-methylnicotinamide and the tests of glucose tolerance, insulin tolerance and liver function. These will be discussed below.

Beginning on the first day of the diet, the subjects had a ketosis, a two-plus positive nitroprusside reaction in the urine which increased to a four-plus from the fifth to the ninth days, and it was not until the third day after the diet that they showed a negative nitroprusside reaction again. This ketosis was shown equally by the subjects who ate the most and the least (G.S and A.R.).

The excretion of N^1-methylnicotinamide increased in the experimental subjects from 0.44 mg per hour to 0.96 mg per hour but the controls stayed practically constant at 0.32 before to 0.42 mg per hour during the experimental period. It was not an effect of starvation as shown by subject G.S. who kept in caloric balance, but was probably due to the large intake of protein.

The urinary excretion of nitrogen averaged 173 gm (1080 gm protein) per person for the 9-day period, the highest daily output being 24.4 gm and the lowest 14.2 gm, which was still high. On the other hand the average nitrogen intake was 103 gm per person (644 gm protein) which was a deficit of 70 gm nitrogen during the whole experimental period. Thus even though the subjects took in reasonable amounts of protein (average 50 gm/day omitting subject G.S. or 69 gm/day including G.S.), all except G.S. still burned their body protein at the average rate of 49 gm per day to help supply their caloric deficiency. The only subject (G.S.) who did not have a caloric deficiency ate 265 gm of protein per day and did not use any of his body nitrogen; in fact he had a positive nitrogen balance.

There were significant changes in both the insulin and glucose tolerance tests (tables 4 and 5). During the period of the diet the blood glucose of the pemmican group did not fall to abnormally low levels either in the basal state or after 2.5

units of crystalline zinc insulin were injected intravenously, but the depression after insulin was definitely prolonged. The most interesting part of the insulin tolerance tests was the reactions that the subjects showed during the dietary period. Whereas no one had any marked symptoms before or after the diet, five of the eight men who had insulin tolerance tests

TABLE 4

Insulin tolerance tests.

GROUP	BLOOD GLUCOSE (MG/100 ML)						
	Fasting	20 min.	25 min.	30 min.	35 min.	45 min.	60 min.
Pemmican							
Before diet	111	83	80	83	93	106	105
Test period	107	77	74	70	80	85	85
After diet	119	83	79	85	87	106	107
Control							
Before diet	106	76	80	88	92	93	96
Test period	108	73	75	83	90	102	106
After diet	112	66	72	86	96	105	106

TABLE 5

Glucose tolerance tests. (All subjects received 75 gm of glucose).

TIME OF TEST	BLOOD GLUCOSE (MG/100 ML)					BLOOD GLUCOSE (MG/100 ML)				
	Fasting	30 min.	60 min.	120 min.	180 min.	Fasting	30 min.	60 min.	120 min.	180 min.
	Pemmican					*Control*				
Before diet	99	168	168	117	102	100	159	145	136	100
Test period	108	173	186	128	104	110	146	135	117	100
After diet	106	159	135	110	113	121	180	148	124	103

during the diet complained of faintness, light headedness, dizziness, hunger pains and nausea. The subjects became pale, sweated considerably and were noticeably glassy-eyed. Each subject received 2.5 units of crystalline zinc insulin intravenously, except W.F. and P.K. who both received 1.6 units. Even with such small doses W.F. had quite a bad reaction, which included nausea, faintness and hunger pains.

The results of the glucose tolerance tests were a surprise to all, for we had expected to see the subjects, on being given the glucose solution during the period on this diet, perk up and become happy and active. Instead, no subject detected any beneficial effects. The blood sugar curves during the glucose tolerance tests were significantly higher in the pemmican group.

TABLE 6

Liver tolerance test.[1]

TIME OF TEST	DYE RETAINED (%)			DYE RETAINED (%)		
	15 min.	30 min.	45 min.	15 min.	30 min.	45 min.
	Pemmican			*Control*		
Before diet	29	12	5	21	9	7
Test period	50	20	12	30	10	5
After diet	29	9	6	24	6	5

[1] 5 mg Bromsulfalein intravenously per kg of body weight.

The ketonuria, which did not disappear until 3 days after the diet was caused not only by starvation but also by the high fat and very low carbohydrate diet. This was shown very clearly during a glucose tolerance test in two different subjects. The nitroprusside reaction was a four-plus before drinking 75 gm of glucose in solution but in 1 hour in one case and 2 hours in another the reaction was negative. We will discuss the ketonuria again later in the paper.

The liver function test during the test period showed a significant increase in the retention of the dye at 15, 30 and 45 minutes (table 6).

DISCUSSION

For practical purposes, the present work supports the contention that pemmican is unsuitable for a field ration if used as the sole component.

At the beginning of the experiment, all the subjects seemed sure that they would have no trouble eating the pemmican, for at first it did not taste too badly when eaten slightly warmed and in small amounts, but this method was soon given up be-

cause it began to be very nauseating. The idea of eating it in the form of a stew also seemed to be good, but here again the novelty wore off within 2 days because the taste of the meat became more and more repulsive as days went by and as a result the amount consumed per person decreased gradually so that on the last day the average consumption was 94 gm (660 cal.).

Kark, Johnson and Lewis ('45) made a thorough search of the literature of explorers in cold climates and reported that there was no clear evidence that men had ever lived successfully on pemmican alone. It was always supplemented with biscuits and cereals and whatever other foods were available. The pemmican was commonly saved until the end, either because it was not liked or possibly because it was the most concentrated and expensive food. Stefanssen ('44), on the other hand, states that a fair test of pemmican should take at least 2 weeks, and in that time a person will grow to like it, but we found that the pemmican was edible in only very small amounts, not sufficient to keep a man in good condition for a long time. We had hoped to acquire a taste for it as days went by but instead it became more and more difficult to eat as time went on. The one subject who ate the most had to force himself through the last half of the experimental period. However, Peary ('17) and Priestley ('15) were enthusiastic about pemmican as the major component of a ration.

Each night the subjects in response to questions concerning subjective feelings and cravings agreed that they would much rather have almost anything to eat in place of pemmican. Many mentioned carbohydrate food but meats were also mentioned occasionally and there was no clear craving for carbohydrate. All had had visions of eating enormous quantities of food on the first meal after the diet, but when this hoped for meal at last came, almost everyone complained that he was filled and satisfied too soon. The average caloric consumption of the subjects at this meal was 1380 cal. and one of the most striking features was that all men ate large amounts of bacon and butter although carbohydrate food was equally available.

This suggests that people in general will eat fat that is naturally in food, but will not eat it in the form of pemmican.

The subjects, after having lost so much weight during the diet, retained an average of 1300 ml of fluids on the first day on a normal diet. We feel that the high fat had a tendency to slightly dehydrate the men, as shown in the case of G.S., who ate large quantities of the pemmican. However, our data are not adequate to differentiate between the effects of high fat and the effects of low chloride upon the water balance. G.S. still lost 3.0 kg in the 9-day period, but all of it was water because the calculated weight change from daily caloric intake and daily expenditure was plus 0.2 kg. This would make his water loss 3.2 kg. G.S. in contrast to the other subjects had slightly more than regained his normal weight by the third day on a normal diet. The weight loss of the whole group, calculated from their estimated caloric deficiency, was 1.7 kg which subtracted from the observed loss of 5.9 kg leaves 4.2 kg due to loss of fluid. The weight regained by the whole group by the third day on a normal diet was only 3.6 kg.

The general physiological findings of the present study confirm and extend the conclusions of Kark, Johnson and Lewis ('45). The biochemical abnormalities produced in our subjects by the high fat diet even in the one who ate the most, included changes in water and salt balance, liver function and glucose and insulin tolerance tests. While the subjects showed a marked ketosis, as might well be expected on this type of diet, it is not clear that this ketosis was detrimental. Heinbecker ('28) has shown that Eskimos on a meat diet do not show ketosis. Our subjects showed no change in scores in the physical fitness tests except for the initial 2 days but their daily work output was small and caloric deficits affect physical fitness most strikingly in men who are working hard.

There was unusually high sensitivity to insulin in five of the eight subjects on pemmican. In the literature very little is said about sensitivity to insulin on a high fat diet, but Himsworth ('34a) states that on a high fat diet the sensitivity to insulin is low and that subsistence on such a diet retards

and diminishes the action of insulin upon the blood sugar. Wishnofsky, Kane and Spitz ('37) conclude that fat does not require insulin for its metabolism for it does not inhibit or retard the action of insulin. Riesser ('42) also states that a protein and fat diet produces a greater decrease in sensitivity to insulin shock than a diet consisting partly or largely of carbohydrate. Some work has been done on the sensitivity to insulin in starvation. Selye ('40) in experiments with rats, states that although fasting progressively increases the insulin sensitivity, there is a transitory period after about 2 days of starvation during which the insulin sensitivity is very low. Gigante ('35) in experiments with starving pigeons, states that the insulin resistance shows a sudden decrease at the point of hunger crisis, usually ending in convulsions. We cannot agree with Himsworth, Wishnofsky, and Riesser, for one of our subjects, G.S., who ate large quantities of the high fat diet, had a bad reaction during the insulin tolerance tests. Another subject, R.W., who practically starved the last 4 days, had reactions that were so severe that glucose had to be administered. To conclude, a high fat diet as well as a semi-starved condition, increased sensitivity to insulin as judged by the reactions other than the fall in blood sugar which fall was not increased though it was prolonged.

The glucose tolerance curves during the dietary regime which were significantly higher are in agreement with those of Himsworth ('34b), Threadwell, King, Babb and Tidwell ('42), and Greene and Swanson ('40), who all state that a high fat diet produces a decrease in sugar tolerance. Wilson ('39) states that the glucose tolerance curve has been employed successfully to detect early deficiencies in liver function. The curve in such cases is high and its fall is delayed and liver extract lowers it towards the normal.

There was increased retention of bromsulfalein dye during the experimental diet. Abnormal retention of the dye has been interpreted as evidence of liver dysfunction (Rosenthal and White, '25; Bulmer, '28; Foley, '30; Macdonald, '38; Helm and Machella, '42). There is general agreement that a

retention of over 5% at 30 minutes is abnormal. One case of Robertson, Swalm and Konzelmann ('32) showed 100% retention and the interpretation was made that an important factor may have been dehydration and decrease in blood volume. In the present experiments there is evidence of changed liver function, not necessarily dysfunction. A hypothesis to explain the findings is that on a high fat diet, the mechanisms for regulating glycogen in the liver are altered in such a way that a given dose of insulin keeps the blood sugar at a low level for an abnormally long time. The bromsulfalein test confirms the alteration of liver function.

The urinary and serum chlorides, which were significantly low at the end of the diet, showed that even though a man ate more than his share of the pemmican daily, as in the case of G.S. who ate from 2800 to 4720 cal., the salt content was insufficient to keep the average man in salt balance with a satisfactory margin of safety over a long period of time. At the end of the diet G.S. was excreting only 5.7 gm of salt per day and his serum chloride had dropped from 106 to 100 milliequivalents per liter.

SUMMARY

A group of eight men living in a cool environment and doing work consisting mostly of laboratory procedures subsisted for 9 days on a high fat diet (pemmican) providing 71% of the calories from beef fat and 2% from carbohydrate. Four controls subsisted on a diet adequate in all respects and providing 30% of the calories from fat.

The utility of pemmican alone as a field ration for ordinary men was very poor because of the inability of all but one subject to eat enough of it. Morale deteriorated on the diet and most of the men resigned themselves to semi-starvation for the duration of the diet, mainly because of the nauseating taste. Nevertheless, scores in a physical fitness test remained practically constant.

Significant biochemical and physiological changes occurred, even in the one man who ate adequate amounts of the pemmican. These included: (a) an average weight loss of 5.9 kg,

much of it water; (b) change in water balance, with loss of body water; (c) salt depletion as measured by serum and urinary chlorides; (d) marked ketonuria; (e) alteration in the glucose tolerance curve, with prolongation of the rise but without alteration of the maximum; (f) alteration in tolerance to a given dose of insulin with much increased physiological reaction and prolongation of the decrease without alteration in the minimum; (g) increased retention of bromsulfalein. All of the above abnormalities were repaired in 3 days of normal diet.

Measurements showing no significant changes include: (a) serum protein; (b) serum non-protein nitrogen; (c) serum ascorbic acid, (d) serum cholesterol; (e) fasting blood glucose; (f) urinary excretion of thiamine, riboflavin and ascorbic acid; (g) basal metabolic rate; (h) phenolsulfonphthalein test of kidney function.

This work should not be taken to apply to all high fat diets, but at this point we do not know why there appears to be a difference between a diet of pemmican and a diet of fresh meat and fat.

ACKNOWLEDGMENTS

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THE EFFECT OF EXCESS DIETARY CALCIUM ON LONGEVITY AND TISSUE CALCIUM IN THE ALBINO RAT[1]

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In a series of articles, it has been shown by Sherman and associates that the calcium content of the body of the growing rat is dependent upon the calcium content of the food supply, at least up to a content of 0.64% and possibly somewhat higher (Toepfer and Sherman, '36; Lanford, Campbell and Sherman, '41). In a later article (Briwa and Sherman, '41), it is concluded from analyses of well over 400 experimental animals sacrificed at 28 days of age "that among normal growing individuals of a given sex, having the same hereditary and nutritional background, age is the predominant determining factor in the increasing percentage of calcium in the body."

In another phase of the same series of studies, Campbell, Pearson and Sherman ('43) demonstrated an improvement in the longevity of rats receiving a diet containing 0.34% of calcium as compared with one containing only 0.20%, in both males and unmated females. Van Duyne and others ('41) claim to have shown that further increases in the concentration of dietary calcium up to 0.64, or possibly 0.80%, "gave best results in permanent nutritional well-being, as shown by life-time experiments in three generations of rats main-

[1] The investigation reported in this paper was aided by funds contributed by the American Dry Milk Institute, Inc., of Chicago, Illinois.

[2] Now with the Golden State Co., Limited, of San Francisco, California.

tained under laboratory conditions." The basal diet in both of these investigations was demonstrably inadequate in riboflavin and vitamin A. On a more favorable basal diet, calcium levels of 0.34, 0.48 and 0.64% induced equally good records throughout the life histories of the experimental animals (Campbell and Sherman, '45).

The experiments thus briefly reviewed are of fundamental importance in nutrition. If they may be criticized at all, it may be on the score that the feeding of a constant diet throughout the life of an animal is not a sufficiently realistic nutritional endeavor if the results obtained are to be applied to human nutrition. Infants, children and adults are not nourished in this manner, nor is such a system one that can be recommended because of the changing nutritive requirements with age and with such functions as growth, reproduction and lactation. Specifically, these experiments furnish no information on the very practical question whether in adult life, and regardless of the special nutritive requirements of gestation and lactation, any advantage, or any disadvantage, accrues from an intake of calcium obviously in great excess of current requirements. Thus, an adult rat may be brought into calcium equilibrium on a diet containing only 0.03% of calcium [3] so that the requirement for continued adult maintenance can hardly be much more than this.

The tissue changes accompanying senescence in man seem to involve rather generally an increase in calcium content (Simms and Stolman, '37) particularly in the hyaline cartilage (Falconer, '38). Sclerosis of the large arteries involves deposition of calcium salts similar in composition to the salts of bone (Meeker and Kesten, '36), while deposition of calcium phosphates in the soft tissues accompanies many types of senile degeneration and disease (Frondel and Prien, '46). Such age changes have been noted in the tissues of rats also by Barnes ('42). Their occurrence is accentuated by retardation of growth (Hummel and Barnes, '38), by the administration of massive doses of irradiated ergosterol (Ham and Lewis, '34),

[3] According to data secured in this laboratory by Dr. John B. Longwell.

and by an excess of calcium and phosphorus in acid-producing diets (Stephens and Barr, '33).

It seemed important to study further the effect of high-calcium diets, especially in adult life, on the occurrence of soft-tissue calcification, and particularly on longevity in the albino rat. If senile degeneration with its concomitant calcification of the soft tissues is dependent solely upon the local initiation of metabolic changes, then an intake of dietary calcium in great excess of current needs will create no hazard to health and will not shorten life. But if these degenerative changes are accelerated by an excessive intake of calcium, then the calcium intake should be lowered in old age and such high-calcium foods as milk and dairy products should be consumed in moderation. It was the purpose of the investigation to be described in this report to throw further light upon this highly practical problem in adult nutrition.

PLAN OF THE EXPERIMENT

Twenty-four trios of rats, twelve trios of male rats and twelve trios of female rats, were fed from shortly after weaning (initial weights of 33 to 64 gm, averaging 45 gm) to natural death on three experimental diets designed to contain liberal amounts of all essential nutrients and to differ only in their contents of calcium and of phosphorus. These differing contents of the diets in the two mineral elements were secured by adding appropriate amounts of calcium carbonate or of dicalcium phosphate to a basal diet consisting of casein 17, dried liver 5, dried yeast 10, salts [4] 2, corn oil 10, sucrose 10, and whole wheat 46. After 43 weeks, the casein in this basal diet was reduced from 17 to 5% and the whole wheat raised from 46 to 58%.

During the first 43 weeks of feeding, Diet A consisted of the basal ration supplemented with calcium carbonate to raise the calcium content to approximately 0.6%. Diets B and C included supplements of dicalcium phosphate to raise the

[4] A Ca- and P-free mixture containing all of the other mineral elements known to be essential in animal nutrition.

calcium content to approximately 1.0%. At the end of 43 weeks, the supplement of calcium carbonate was eliminated from Diet A. The calcium content of Diet B was reduced to approximately 0.7%, using a calcium carbonate rather than a calcium phosphate supplement. Diet C was unchanged. Throughout the experiment each rat received 3 drops of cod liver oil daily and 3 drops of wheat germ oil weekly.

The contents of calcium, phosphorus and ash in the three diets and in the two divisions of the experiment, by actual analysis, are summarized in table 1. It is evident that the rats on Diet A were raised on an adequate but not excessive

TABLE 1

Chemical composition of the experimental diets.

PERIOD COVERED	DIET A			DIET B			DIET C		
	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash
	%	%	%	%	%	%	%	%	%
First 43 weeks	0.59	0.56	4.15	0.98	0.86	5.45	0.98	0.86	5.45
Remainder of experiment	0.22	0.48	3.52	0.69	0.51	4.58	1.06	0.85	5.26

plane of calcium nutrition, and then when growth was completed, or shortly thereafter, the plane of calcium nutrition was lowered in conformance with lowered requirements. The rats on Diets B and C were raised on an excessive plane of calcium nutrition (about 1% in the diet). In early adult life, the calcium level of the rats on Diet B was lowered to about 0.7%, while that of the rats on Diet C was maintained at the 1.0% level throughout life. The latter rats were, thus, fed excessive amounts of calcium throughout life, especially during adulthood.

The rats were litter mates in 18 of the 24 trios, and in all trios were selected for practical equality in initial body weight. Within each trio, one rat received Diet A, one Diet B and one Diet C. Trio mates were fed equal amounts of organic matter in their respective diets, due account being taken of differences between diets in content of ash, until the death of one of the trio mates. The remaining two rats were then continued

as a pair with equal intakes of organic matter until one died, when the remaining rat was continued on an amount of food chosen voluntarily. This plan of feeding was adopted in an attempt to secure a more exact comparison of the three calcium regimes, uncomplicated in so far as possible by differences in the consumption of calories, protein, etc. If these differences had been allowed to develop, whether as subsidiary effects of the experimental diets or merely as expressions of individuality among the members of the same diet groups, the results secured could not have been so surely attributed to the imposed differences in calcium nutrition and the experimental error in average data, originating in the uncontrolled factors of the investigation, could not have been kept at a minimum, or so it seemed.

Throughout the experiment, the rats were kept in an air-conditioned room, maintained at a temperature of $80 \pm 2°$F., and a relative humidity of $50 \pm 5\%$.

It is realized that the chemical composition of the bodies of animals allowed to live until natural death may be determined not only by the dietary regime upon which they have subsisted, but also upon the nature and the duration of the terminal illness. For this reason, a study of the effect of diet upon the life span and a study of the effect of diet upon tissue composition are to a degree mutually incompatible. However, it was considered worth while to determine the calcium content of certain selected tissues of some of the experimental rats after natural death, as well as the ash content of selected bones. The effect of morbidity on these results will presumably be minimized if they are expressed on the moisture-free basis, or, in the case of the bones, on the moisture- and fat-free basis.

EXPERIMENTAL RESULTS

Rate of growth

The rats in all diet groups increased in body size at a satisfactory rate considering the type of dietary control imposed in order to permit the most significant comparison of the

three dietary regimes. The average data summarized in table 2 show that mature weights were attained in from 40 to 60 weeks. They also reveal no significant differences in rate of growth among the three diet groups within the two sexes. The males continued to increase slowly in body weight up to the one hundredth week to an average weight of 330 to 340 gm, presumably due to fat deposition, and then, as a group, decreased rather precipitously until death. The females maintained an average body weight of 200 to 240 gm from the

TABLE 2

Average body weights in grams at different periods in the experiment.

TIME INTERVAL AFTER:	DIET A	DIET B	DIET C	TIME INTERVAL AFTER:	DIET A	DIET B	DIET C
weeks		MALES		*weeks*		FEMALES	
10	197	202	199	10	158	163	160
20	246	254	249	20	188	188	186
30	282	288	279	30	199	201	193
40	278	293	280	40	205	207(11)[1]	203
50	287(11)[1]	296	290(10)[1]	50	195	204(11)[1]	194
60	311(9)[1]	315(11)[1]	287(10)[1]	60	208(11)[1]	215(7)[1]	203(10)[1]

[1] The numbers in parentheses denote the number of rats surviving at the indicated time when the original group size of 12 has been reduced by mortality.

thirtieth to the ninetieth week of the experiment, before the pre-mortal decrease in average weight supervened. The females on Diets B and C exhibited a greater average tendency to fatten in adult life than the females on Diet A, but the significance of these changes in group average weights in later life is obscured by the decreasing size of groups due to increasing mortality.

In table 3 are given the average body weights of the two surviving members of the trios in both sexes at the time of death of the first member of the trio. In ten of the twenty-four trios, the first death occurred in the rat subsisting on Diet C; in eight trios, the first death occurred in the rat on Diet B; and in six it occurred in the rat on Diet A. The average body

weights of the remaining two rats in each trio at the time of death of the first trio mate are not greatly different between the diet groups involved, although the weight advantage on the average favored in each case the rat on the more liberal calcium regime. Only one of these diet differences was sufficiently consistent among the pairs of rats involved and involved a sufficient number of pairs to be significant statistically, i.e., the difference between Diet A and Diet B in the female rats, averaging 20 gm ($P = 0.016$) (Student, '25).

TABLE 3

Average body weights of paired rats at death of first rat in trio.

NO. CASES	AVERAGE AGE AT DEATH OF FIRST RAT IN TRIO DAYS	AVERAGE BODY WEIGHTS			NO. CASES	AVERAGE AGE AT DEATH OF FIRST RAT IN TRIO DAYS	AVERAGE BODY WEIGHTS		
		Diet A	Diet B	Diet C			Diet A	Diet B	Diet C
		MALES					FEMALES		
5	498	285	307	...	5	465	197	217	...
2	473	224	...	237	6	364	199	...	208
5	577	...	259	283	1	601	...	248	250

Longevity

In order to minimize the spread of respiratory infections among the experimental rats, those obviously infected were separated from the rest and were always handled and fed last each day. The causes of death were largely respiratory in nature.

The average age at death in each diet and sex group is given in table 4. The table also includes the coefficients of

TABLE 4

Average age at death in days with coefficients of variation.

SEX	DIET A	DIET B	DIET C	SEX	DIET A	DIET B	DIET C
	Averages				*Coefficients of variation*		
Males	641	756	630	Males	32	29	30
Females	627	563	632	Females	20	36	31

variation in age at death. It is evident that age at death in populations of rats subsisting on the same diet is a very variable function, with an average coefficient of variation of about 30. The standard deviation in age at death for all seventy-two rats, calculated from the averages of the respective diet groups is 185 days, varying from 126 to 218 for the six diet and sex groups. This value may be compared with values of 170 and 138 days for the two groups in the longevity experiments of Campbell, Pearson and Sherman ('43) and of 132, 200, 139, and 168 for the diet and sex groups of Van Duyne et al. ('41). Such extreme variation increases the difficulty in establishing the statistical significance of diet (and other) effects on the longevity of the rat.

TABLE 5

Significance of diet differences in age at death within trios expressed as the probability of a chance outcome.

	DIET A	DIET B	DIET C	DIET A	DIET B	DIET C
		MALES			FEMALES	
Diet A	....	0.066	0.44	...	0.19	0.47
Diet B	0.066	....	0.063	0.19	...	0.23
Diet C	0.44	0.063	....	0.47	0.23	...

In an attempt to establish the statistical significance of the average differences in longevity among the different diet groups, differences in age at death among the three diet regimes between trio mates were analyzed according to the method of Student ('25). The statistics summarized in table 5 are the probabilities that a fortuitous combination of the uncontrolled factors in the experiment would have caused as great or greater average differences (of the same sign) among comparable diet groups than those actually observed. If the probability of a chance outcome is 0.03 or less it may be considered so small that it may be neglected. In such a case it may reasonably be concluded that the imposed difference in calcium nutrition between the diet groups involved was the predominant factor in determining the group divergence.

Among the female diet groups, none of the differences approached statistical significance. Among the male diet groups, the rats subsisting on Diet B stood out among the other groups as the longer lived, on the average, but the probabilities of 0.063 and 0.066 are still too large to be neglected. The suggestion contained in these low probabilities, that Diet B may have favored the greatest length of life in the male rats, must be tempered by the fact that, among the female rats, Diet B induced the shortest average life span of all three diets.

If the data are analyzed as unpaired groups according to Fisher's ('44, p. 122) modification of Student's method, somewhat larger probabilities of a chance outcome are secured among the male groups, and equal, or somewhat smaller probabilities among the female groups. The method of food intake control practiced in this experiment has, thus, not contributed appreciably to the effectiveness of the experiment.

The calcium content of the tissues

It is not believed that the calcium content of the fresh carcass or of the fresh tissues in experimental animals after natural death is of great significance, because of the probable disturbances in water balance associated with the terminal disease. Hence, all calcium analyses are expressed on the dry weight of tissue, or on the tissue ash. Even with these values there is no assurance that the terminal illness, with its inevitable disturbance in appetite, may not have modified the results.

Table 6 summarizes the average per cent ash in the dry defatted bones (leg bones) and the average per cent calcium in the bone ash and the carcass ash. The differences between comparable group averages are small and are all statistically insignificant.

The average calcium contents of the samples of dry liver, kidney, heart and muscle are to be found in table 7. The averages for comparable groups suggest a tendency for the excessive-calcium group (Diet C) to possess soft tissues con-

TABLE 6

Average mineral content of the bones and carcass ash.

NUMBER OF CASES	SEX	DIET A	DIET B	DIET C
		%	%	%
		Ash in dry defatted bones		
7 to 8	m	60.7	60.6	61.8
5 or 6	f	61.9	60.2	61.4
13 or 14	both	61.2	60.4	61.7
		Calcium in bone ash		
7	m	36.1	37.6	37.2
4	f	37.8	38.0	37.6
	both	36.7	37.7	37.4
		Calcium in carcass ash		
6 or 7	m	27.9	28.3	28.6
4	f	29.0	27.6	29.0
	both	28.4	28.1	28.8

TABLE 7

Average per cent calcium in the soft tissues expressed on the dry basis.

TISSUE	SEX	NUMBER OF CASES	DIET A	DIET B	DIET C
Liver	m	6 to 9	0.038	0.040	0.042
Liver	f	3 or 5	0.040	0.040	0.045
Liver	both	11 or 13	0.039	0.040	0.043
Kidney	m	6 to 9	0.065	0.062	0.090
Kidney	f	4 or 5	0.090	0.064	0.080
Kidney	both	11 to 13	0.073	0.062	0.085
Heart	m	6 to 9	0.048	0.048	0.076
Heart	f	3 or 4	0.097	0.040	0.050
Heart	both	9 to 13	0.063	0.045	0 068
Muscle	m	5 to 8	0.041	0.032	0.050
Muscle	f	4 or 5	0.034	0.042	0.052
Muscle	both	10 or 12	0.039	0.036	0.051
All tissues	both	.....	0.054	0.046	0.062

taining the greatest degree of calcification. When the individual trios are examined, this tendency is almost completely obscured by the intra-trio variation in the case of the kidney and the liver. It is still quite evident for the heart and especially the muscle samples, however, and in the latter case the tendency is quite significant as between Diets C and B ($P = 0.025$), but merely suggestive as between Diets C and A ($P = 0.10$).

The individual data on the calcium content of the soft tissues, expressed on the dry basis, reveal no correlation with age at death. However, only three of the rats whose tissues were submitted to analysis died at ages less than 1 year, i.e., 307, 325 and 359 days. Also, no evident correlation exists among the calcium contents of the dry soft tissues for the same rat, indicating that a relatively high content of calcium in the dry liver, for example, gives no assurance that the other soft tissues in the same carcass will also show relatively high contents of calcium. This situation would indicate that calcium metastasis is primarily the result of local conditions in the soft tissues, rather than of systemic conditions.

SUMMARY AND CONCLUSIONS

An experiment on twenty-four trios of rats was undertaken to determine the effect of excessive calcium nutrition, especially in adult life, on growth, longevity and calcification of the tissues, such as occurs in many of the soft tissues during senescence. Twenty-four rats were carried from weaning to death on a diet containing about 1% of calcium. Another group of the same size subsisted on a diet containing 1% of calcium during the growing period and then only 0.7% for the remainder of their lives. A third group was raised on a 0.6% calcium level and then on a 0.22% level. The diets were otherwise satisfactory especially with reference to vitamin D and phosphorus.

The rate of growth was not appreciably affected by the different dietary regimes, though among the female rats the more liberal calcium diet was associated with a greater degree

of adult "growth," probably consisting mainly of fat deposition.

The data on longevity do not reveal any significant diet effects among either male or female rats. The great variability in age at death among rats on the same diet, represented by a standard deviation of 185 days, testifies to the difficulty of establishing the statistical significance of imposed experimental conditions in longevity studies.

A slight tendency for somewhat greater calcification of the soft tissues, especially of the muscles, on the highest calcium regime studied, is suggested by the data presented, though complications induced by the terminal illness render interpretation of these differences difficult.

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